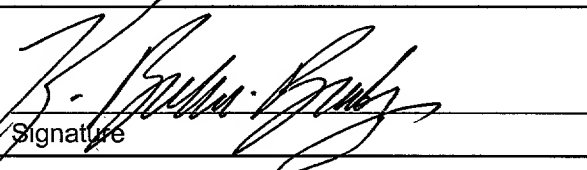
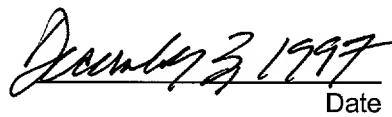


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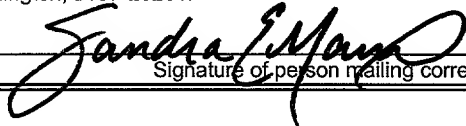
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APPLICATION
FOR
UNITED STATES LETTERS PATENT

APPLICANT : H. ROBERT HORVITZ, JUNYING YUAN, SHAI SHAHAM
TITLE : CLONING, SEQUENCING AND CHARACTERIZATION OF
TWO CELL DEATH GENES AND USES THEREFOR

CLONING, SEQUENCING AND CHARACTERIZATION
OF TWO CELL DEATH GENES AND USES THEREFOR

Government Funding

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5 GM24663 and GM24943 from the U.S. Public Health
Service. The U.S. Government has certain rights in the
invention.

Related Application

This application is a continuation-in-part of USSN
10 07/897,788, entitled "Cloning, Sequencing and
Characterization of Two Cell Death Genes and Uses
Therefor" by H. Robert Horvitz, Junying Yuan, and Shai
Shaham, filed June 12, 1992. The teachings of USSN
07/897,788 are incorporated by reference.

15 Background

Cell death is a fundamental aspect of animal
development. Many cells die during the normal develop-
ment of both vertebrates (Glucksmann, *Biol. Rev.*
Cambridge Philos. Soc. 26:59-86 (1951)) and inverte-
20 brates (Truman, *Ann. Rev. Neurosci.* 7:171-188 (1984)).
These deaths appear to function in morphogenesis,
metamorphosis and tissue homeostasis, as well as in the
generation of neuronal specificity and sexual
dimorphism (reviewed by Ellis et al., *Ann. Rev. Cell*
25 *Biol.* 7:663-698 (1991)). An understanding of the
mechanisms that cause cells to die and that specify
which cells are to live and which cells are to die is
essential for an understanding of animal development.

The nematode *Caenorhabditis elegans* is an
30 appropriate organism for analyzing naturally-occurring
or programmed cell death (Horvitz et al., *Neurosci.*
Comment. 1:56-65 (1982)). The generation of the 959

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5 somatic cells of the adult *C. elegans* hermaphrodite is
accompanied by the generation and subsequent deaths of
an additional 131 cells (Sulston and Horvitz, *Dev.*
Biol. 82:110-156 (1977); Sulston et al., *Dev. Biol.*
10 100:64-119 (1982)). The morphology of cells undergoing
programmed cell death in *C. elegans* has been described
at both the light and electron microscopic levels
(Sulston and Horvitz, *Dev. Biol.* 82:100-156 (1977);
Robertson and Thomson, *J. Embryol. Exp. Morph.* 67:89-
10 100 (1982)).

Many genes that affect *C. elegans* programmed cell
death have been identified (reviewed by Ellis et al.,
Ann. Rev. Cell Biol. 7:663-698 (1991)). The activities
of two of these genes, *ced-3* and *ced-4*, are required
15 for the onset of almost all *C. elegans* programmed cell
deaths (Ellis and Horvitz, *Cell* 44:817-829 (1986)).
When the activity of either *ced-3* or *ced-4* is
eliminated, cells that would normally die instead
survive and can differentiate into recognizable cell
20 types and even function (Ellis and Horvitz, *Cell*
44:817-829 (1986); Avery and Horvitz, *Cell* 51:1071-1078
(1987); White et al., *Phil. Trans. R. Soc. Lond. B.*
331:263-271 (1991)). Genetic mosaic analyses have
indicated that the *ced-3* and *ced-4* genes most likely
25 act in a cell autonomous manner within dying cells,
suggesting that the products of these genes are
expressed within dying cells and either are cytotoxic
molecules or control the activities of cytotoxic
molecules (Yuan and Horvitz, *Dev. Biol.* 138:33-41
30 (1990)).

Summary of the Invention

This invention relates to genes shown to be
essential for programmed cell death, referred to herein
as cell death genes, to their encoded products (RNA and

5 invertebrates, and possibly, microbes and plants, are described. Agents which mimic or affect the activity of cell death genes and methods for identifying these agents are also described. Bioassays which detect the activity of cell death genes and which are useful for
10 identifying cell death genes, for testing the effect of mutations in cell death genes, and for identifying agents which mimic or affect the activity of cell death genes are also provided. This invention further relates to methods for altering (increasing or
15 decreasing) the activity of the cell death genes or their encoded products in cells and, thus, for altering the proliferative capacity or longevity of a cell population or organism.

As described herein, the *ced-3* and *ced-4* genes can be used to identify structurally related genes from a variety of sources. Some of these related genes are likely to also function as cell death genes.

30 Structural comparison of related cell death genes, as
well as mutational analysis, can provide insights into
functionally important regions or features of cell
death genes and gene products. This information is
useful in the design of agents which mimic or which
35 alter the activity of cell death genes.

This invention further provides methods and agents for altering (increasing or decreasing) the occurrence of cell death in a cell population or organism. Methods and agents, described herein, which decrease cell death are potentially useful for treatment (therapeutic and preventive) of disorders and conditions characterized by cell deaths, including myocardial infarction, stroke, traumatic brain injury, degenerative diseases (e.g., Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and Duchenne's muscular dystrophy), viral and other types of pathogenic infection (e.g., human immunodeficiency virus, HIV), aging and hair loss. Methods and agents which increase cell death are also provided and are potentially useful for reducing the proliferation or size of cell populations, such as cancerous cells, cells infected with viruses (e.g., HIV) or other infectious agents, cells which produce autoreactive antibodies and hair follicle cells. Such methods and agents may also be used to incapacitate or kill undesired organisms, such as pests, parasites, and recombinant organisms.

Brief Description of the Drawings

Figure 1 shows the genomic organization and nucleotide sequence (Seq. ID #1) of *ced-4* and deduced amino acid sequence (Seq. ID #2). The genomic sequence of the *ced-4* region was obtained from plasmid C10D8-5, which rescues the *ced-4* mutant phenotype. Two likely transcriptional start sites are marked with downward arrows. The start of the cDNA is marked with a solid arrowhead. The positions of eight *ced-4* mutations are indicated by upward arrows. Numbers on the sides indicate nucleotide positions, beginning at the start

of C10D8-5. Numbers under the amino acid sequence indicate codon positions. Vertical lines between nucleotides indicate splice junctions.

Figure 2 shows the genomic structure of the *ced-4* gene and positions of *ced-4* mutations. The sizes of exons and introns are indicated in base pairs (bp). The downward arrows indicate the positions of the Tc4 insertion in the *ced-4(n1416)* mutant and of eight EMS-induced mutations of *ced-4*. The arrow pointing right indicates the direction of transcription. The solid arrowhead indicates the translation initiation site. The open arrowhead indicates the ochre termination codon.

Figure 3 shows the sequence similarities between the Ced-4 protein and some calcium-binding proteins. The consensus sequence of the calcium-binding loop is shown at the top. The positions indicated by X, Y, Z, -X, and -Z correspond to vertices of an octahedron. The numbers above the X, Y, Z, -X and -Z correspond to the positions of the residues within the 29 amino acid EF-hand sequence. Amino acids are indicated by the single letter code. O, amino acid with an oxygen-containing side chain. *, non-conserved amino acid. Positions Y, Z and -X can be any amino acid with oxygen-containing side chains. Position X is usually aspartic acid, and position -Z is usually glutamic acid. Conserved amino acids are shown in bold-face. Deviations from the EF-hand consensus sequence are underlined.

Figure 4 shows the nucleotide sequence (Seq. ID #18) of *ced-3* and deduced amino acid sequence (Seq. ID #19). The genomic sequence of the *ced-3* region was obtained from plasmid pJ107, which rescues the *ced-3* mutant phenotype. The likely translation initiation site is indicated by a solid arrowhead. The SL1 splice

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animals. ++, the number of cell corpses above 10. +, the number of cell corpses below 10 but above 2. -, the number of cell corpses below 2.

Detailed Description of the Invention

5 The *ced-3* and *ced-4* genes of *C. elegans* have been shown to be required for almost all programmed cell deaths in *C. elegans* development (Ellis and Horvitz, *Cell* 44:817-829 (1986)). The present work describes the cloning, sequencing and characterization of these
10 genes. As a result of this work, two genes whose activities are required for cell death, referred to herein as cell death genes, and their encoded products (RNA, polypeptide) are available for a variety of uses. Described below are the cloning and characterization of
15 the *C. elegans ced-4* and *ced-3* genes, methods and probes for identifying structurally related genes, methods for identifying cell death genes from a variety of organisms, methods for identifying agents which mimic or which affect the activity of cell death genes,
20 and methods and agents for altering cell death activity and thus, for altering the occurrence of cell death in a cell population or organism.

The activity of a cell death gene is intended to include the activity of the gene itself and of the encoded products of the gene. Thus, agents and mutations which affect the activity of a gene include those which affect the expression as well as the function of the encoded RNA and protein. The agents may interact with the gene or with the RNA or protein encoded by the gene, or may exert their effect more indirectly.

5 gene, confirming that *ced-4* function is not essential for viability.

Two regions of the inferred Ced-4 protein have sequence similarity to known calcium-binding domains (Kretsinger, *Cold Spring Harbor Symp. Quant. Biol.* 52:499-510 (1987)), suggesting that Ced-4 activity and hence, programmed cell death may be modulated by calcium (see Figure 3 and Example 1). Calcium has been implicated as an essential mediator of cell death in other organisms under a variety of conditions. For example, extracellular calcium is required for glucocorticoid-induced thymocyte death (Cohen and Duke, *J. Immunol.* 132:38-42 (1984)), for the deaths of adult rat hepatocytes induced by certain toxins *in vitro* (Schanne et al., *Science* 206:700-702 (1979)), for agonist-induced muscle degeneration in mice (Leonard and Salpeter, *J. Cell Biol.* 82:811-819 (1979)) and for neuronal cell death caused by oxygen deprivation or excitotoxicity (Coyle et al., *Neurosci. Res. Prog. Bull.* 19:331-427 (1981); Choi, *J. Neurosci.* 7:369-379 (1987), Choi, *Trends Neurosci.* 11:465-469 (1988)). It is possible that programmed cell death is initiated during *C. elegans* development by an increase in intracellular calcium, which activates the Ced-4 protein to become cytotoxic. On the other hand, certain cells seem to be protected against cell death by calcium (e.g., Koike et al., *Proc. Natl. Acad. Sci. USA* 86:6421-6425 (1989); Collins et al., *J. Neurosci.* 11:2582-2587 (1991)), suggesting that increases in intracellular calcium levels may inhibit the activity

of the Ced-4 protein and thereby prevent programmed cell death.

The level of the *ced-4* transcript in eggs is about 20% that of the actin 1 transcript, which is relatively abundant (Edwards and Wood, *Dev. Biol.* 97:375-390 (1983)). This level seems higher than might be expected if *ced-4* were expressed only in dying cells, since in an embryo there are usually no more than two or three cells dying at the same time. These considerations suggest that *ced-4* might be transcribed not only in dying cells but in other cells as well. Perhaps Ced-4 activity, at least during embryonic development, is regulated at a post-transcriptional level. For example, the Ced-4 protein might have to interact with other proteins or other factors (such as calcium) to cause cell death. Since the *ced-3* gene is also essential for programmed cell death in *C. elegans*, one possibility is that the activity of the Ced-4 protein is dependent upon *ced-3* function.

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The *ced-3* Gene

The cloning, sequencing and characterization of the *ced-3* gene are described in Example 2. The *ced-3* gene was cloned by mapping DNA restriction fragment length polymorphisms (RFLPs) and chromosome walking. A 7.5 kb fragment of genomic DNA was shown to complement *ced-3* mutant phenotypes. A 2.8 kb transcript was further identified. The *ced-3* transcript was found to be most abundant in embryos, but was also detected in larvae and young adults, suggesting that *ced-3* is not only expressed in cells undergoing programmed cell death.

A 2.5 kb cDNA corresponding to the *ced-3* mRNA was sequenced. The genomic sequence was also determined (Figure 4; Seq. ID #18) and a comparison with the cDNA

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sequence revealed that the *ced-3* gene has 8 introns which range in size from 54 to 1195 bp (Figure 5A). The four largest introns as well as sequences 5' of the start codon contain repetitive elements, some of which have been previously characterized in non-coding regions of other *C. elegans* genes such as *fem-1* (Spence et al., Cell 60:981-990 (1990)), *lin-12* (J. Yochem, personal communication), and *myoD* (Krause et al., Cell 63:907-919 (1990)). The transcriptional start site was also mapped, and the *ced-3* transcript was found to be trans-spliced to a *C. elegans* splice leader, SL1.

Twelve EMS-induced *ced-3* alleles were also sequenced. Eight of the mutations are missense mutations, two are nonsense mutations, and two are putative splicing mutations (Table 3). The molecular nature of these mutations, together with results of genetic and developmental analyses of nematodes homozygous for these mutations, indicate that, like *ced-4*, *ced-3* function is not essential to viability. In addition, 10 out of the 12 mutations are clustered in the C-terminal region of the gene (Figure 5B), suggesting that this portion of the encoded protein may be important for activity.

The *ced-3* gene encodes a putative protein of 503 amino acids (Figure 4; Seq. ID #19). The protein is very hydrophilic and no significantly hydrophobic region can be found that might be a transmembrane domain (Figure 6). One region of the *ced-3* protein is very rich in serine. Sequence comparison of two additional *ced-3* genes from related nematodes, *C. briggsae* and *C. vulgaris*, suggests that the exact sequence in this serine-rich region may not be important but that the serine-rich feature is (Figure 7; Seq. ID #19-21). This hypothesis is supported by the analysis of *ced-3* mutations: none of 12 EMS-induced

ced-3 mutations is in the serine-rich region (Figure 5B).

The conservation of the serine-rich feature among the *ced-3* genes of different nematodes suggests that the serine-rich region may act in semi-specific protein-protein interactions, similar to acid blobs in transcription factors and basic residues in nuclear localization signals. In all these cases, the exact primary sequence is not important.

It is possible that the serine residues in the Ced-3 and Ced-4 proteins may be targets for a Ser/Thr kinase, and that the activity of these proteins may be regulated post-translationally by protein phosphorylation. McConkey et al. (*J. Immunol.*, 145:1227-1230 (1990)) have shown that phorbol esters, which stimulate protein kinase C, can block the death of cultured thymocytes induced by exposure to Ca^{++} ionophores or glucocorticoids (Wyllie, *Nature* 284:555-556 (1980); Wyllie et al., *J. Path.* 142:67-77 (1984)). It is possible that protein kinase C may inactivate certain cell death proteins by phosphorylation, and thus, inhibit cell death and promote cell proliferation. Several agents that can elevate cytosolic cAMP levels have been shown to induce thymocyte death, suggesting that protein kinase A may also play a role in mediating thymocyte death. Further evidence suggests that abnormal phosphorylation may play a role in the pathogenesis of certain cell-degenerative diseases. For example, abnormal phosphorylation of the microtubule-associated protein Tau is found in the brains of Alzheimer's disease and Down's syndrome patients (Grundke-Iqbal et al., *Proc. Natl. Acad. Sci. USA* 83:4913-4917 (1986); Flament et al., *Brain Res.* 516:15-19 (1990)). Thus, it is possible that phosphorylation may have a role in

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regulating programmed cell death in *C. elegans*. This is consistent with the fairly high levels of *ced-3* and *ced-4* transcripts which suggest that transcription regulation alone may be insufficient to regulate
5 programmed cell death.

Structurally and Functionally Related Genes

As a result of the work described herein, it is possible to identify genes which are structurally
10 and/or functionally related to *ced-3* or *ced-4*. Such genes are expected to be found in a variety of organisms, including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast) and possibly plants.
15 Structurally related genes refer herein to genes which have some structural similarity to the nucleotide sequences (genomic or cDNA) of one or both of the *ced-3* or *ced-4* genes, or whose encoded proteins have some similarity to one or both of the amino acid sequences
20 of the Ced-3 or Ced-4 proteins. Functionally related genes refer to genes which have similar activity to that of *ced-3* and *ced-4* in that they cause cell death. Such genes can be identified by their ability to complement *ced-3* or *ced-4* mutations in bioassays, as
25 described below.

Previous studies are consistent with the hypothesis that genes similar to the *C. elegans ced-3* and *ced-4* genes may be involved in the cell deaths that occur in both vertebrates and invertebrates. Some
30 vertebrate cell deaths share certain characteristics with the programmed cell deaths in *C. elegans* that are controlled by *ced-3* and *ced-4*. For example, up to 14% of the neurons in the chick dorsal root ganglia die immediately after their births, before any signs of
35 differentiation (Carr and Simpson, *Dev. Brain Res.*

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possible that the genes induced in these dying vertebrate and invertebrate cells are cell death genes similar to the *C. elegans* genes *ced-3* and *ced-4*.

Also supporting the hypothesis that cell death in *C. elegans* is mechanistically similar to cell death in vertebrates is the observation that the protein product of the *C. elegans* gene *ced-9* is similar in sequence to the human protein Bcl-2. *ced-9* has been shown to prevent cells from undergoing programmed cell death during nematode development by antagonizing the activities of *ced-3* and *ced-4* (Hengartner, et al., *Nature* 356:494-499 (1992)). The *bcl-2* gene has also been implicated in protecting cells against cell death. It seems likely that the genes and proteins with which *ced-9* and *bcl-2* interact are similar as well.

Genes which are structurally related to *ced-3* or *ced-4* are likely to also act as cell death genes. Structurally related genes can be identified by any number of detection methods which utilize a defined nucleotide or amino acid sequence or antibodies as probes. For example, nucleic acid (DNA or RNA) containing all or part of the *ced-3* or *ced-4* gene can be used as hybridization probes or as polymerase chain reaction (PCR) primers. Degenerate oligonucleotides derived from the amino acid sequence of the Ced-3 or Ced-4 proteins can also be used. Nucleic acid probes can also be based on the consensus sequences of conserved regions of genes or their protein products. In addition, antibodies, both polyclonal and monoclonal, can be raised against the Ced-3 and/or Ced-4 proteins and used as immunoprobes to screen expression libraries of genes.

One strategy for detecting structurally related genes in other organisms is to initially probe animals which are taxonomically closely related to the source

of the probes, for example, probing other worms with a *ced-3* or *ced-4* probe. Closely related species are more likely to possess related genes or gene products which are detected with the probe than more distantly related organisms. Sequences conserved between *ced-3* or *ced-4* and these new genes can then be used to identify similar genes from less closely related species. Furthermore, these new genes provide additional sequences with which to probe the molecules of other animals, some of which may share conserved regions with the new genes or gene products but not with *ced-3*, *ced-4*, or their gene products. This strategy of using structurally related genes in taxonomically closer organisms as stepping stones to genes in more distantly related organisms can be referred to as walking along the taxonomic tree.

Groups of structurally related genes, such as those obtained by using the above-described strategy, can be referred to as gene families. Comparison of members within a gene family, or their encoded products, may indicate functionally important features of the genes or their gene products. Those features which are conserved are likely to be significant for activity. Such conserved sequences can then be used both to identify new members of the gene family and in drug design and screening. For example, as described in Example 2, genes similar to *ced-3* from two other species of nematodes (*C. briggsae* and *C. vulgaris*) were identified and characterized. Serine-rich regions were found in the polypeptides encoded by all three genes. Although the sequence of the serine-rich region was not well conserved, the number of serines was conserved, suggesting that the serine-rich feature, but not the exact sequence of the serine-rich region, is significant for function.

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exogenous cell death gene is underexpressed or inactive can be used to identify agents that activate or increase its activity. Test molecules can be introduced into nematodes by microinjection, diffusion, ingestion, shooting with a particle gun, or other method.

Mutated cell death genes with novel properties may be identified by the above bioassay. For example, constitutively activated or hyperactive cell death genes may be isolated which may be useful as agents to increase cell deaths. Mutations may also produce genes which do not cause cell death but which antagonize the activity of the wild-type gene.

Agents can be obtained from traditional sources, such as extracts (e.g., bacterial, fungal or plant) and compound libraries, or by newer methods of rationale drug design. Information on functionally important regions of the genes or gene products, gained by sequence and/or mutational analysis, as described above, may provide a basis for drug design. The activity of the agents can be verified both by *in vivo* bioassays using nematodes which express various forms of *ced-3*, *ced-4*, or related genes, as described above, and by *in vitro* systems, in which the genes are expressed in cultured cells, or in which isolated or synthetic gene products are tested directly in biochemical experiments. The agents may include all or portions of the *ced-3*, *ced-4*, or related genes, mutated genes, and all or portions of the gene products (RNA, including antisense RNA, and protein), as well as nucleic acid or protein derivatives, such as oligonucleotides and peptides, peptide and non-peptide mimetics, and agonists and antagonists which affect the activity or expression of the cell death genes. The agents can also be portions or derivatives of genes or

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gene products which are not cell death genes but which regulate the expression of, interact with, or otherwise affect the function of cell death genes or gene products.

5 Uses of the Invention

Using the above-described probes and bioassays, the identification and expression of *ced-3*, *ced-4* or related cell death genes in cultured cells, tissues, and whole organisms can be studied to gain insights
10 into their role in development and pathology in various organisms. For example, the detection of abnormalities in the sequence, expression, or activity of a cell death gene or gene product may provide a useful diagnostic for diseases involving cell deaths.

15 This invention further provides means of altering or controlling the activity of a cell death gene in a cell, and, thus, affecting the occurrence of cell death. Activity of the cell death gene can be altered to either increase or decrease cell deaths in a
20 population of cells and, thus, affect the proliferative capacity or longevity of a cell population, organ, or entire organism.

Agents which act as inactivators or antagonists of the activity of *ced-3*, *ced-4*, or other cell death genes
25 can be used to prevent or decrease cell deaths. Such agents are useful for treating (i.e., for both preventive and therapeutic purposes) disorders and conditions characterized by cell deaths, including neural and muscular degenerative diseases, stroke,
30 traumatic brain injury, myocardial infarction, viral (e.g., HIV) and other types of pathogenic infections, as well as cell death associated with normal aging and hair loss. The agent can be delivered to the affected cells by various methods appropriate for the cells or

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organs being treated, including gene therapy. For example, anti-sense RNA encoded by all or a part of a cell death gene which is complementary to the mRNA can be delivered to a population of cells by an appropriate
5 vector, such as a retroviral or adenoviral vector, or an antagonist of cell death activity can be infused into a wound area to limit tissue damage.

Methods and agents which cause or increase cell deaths are also useful, for example, for treating
10 disorders characterized by an abnormally low rate or number of cell deaths or by excessive cell growth, such as neoplastic and other cancerous growth. Such methods and agents are also useful for controlling or eliminating cell populations, such as cells infected
15 with viruses (e.g., HIV) or other infectious agents, cells producing autoreactive antibodies, and hair follicle cells. In addition, methods and agents which increase cell death can be used to kill or incapacitate undesired organisms, such as pests, parasites and
20 genetically engineered organisms. All or portions of *ced-3*, *ced-4*, or related cell death genes, active mutant genes, their encoded products, agents which mimic the activity of cell death genes, and activators and agonists of cell death genes can be used for this
25 purpose.

For example, cell death genes can be used to kill cells infected with the human immunodeficiency virus (HIV), and thus, prevent or limit HIV infection in an individual. A recombinant gene can be constructed, in
30 which a cell death gene is under the control of a viral promoter which is specifically activated by a viral protein; the recombinant gene is introduced into HIV infected cells. HIV-infected cells containing the viral activator protein would express the cell death

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gene product and be killed, and uninfected cells would be unaffected.

Alternatively, an antagonist of *ced-3* or *ced-4* activity (such as antisense RNA) can be expressed under the control of a viral-specific promoter and in this way, be used to prevent the cell death associated with viral (e.g., HIV) infection.

In another example, cell death genes can be used as suicide genes for biological containment purposes. Genetic engineering of suicide genes into recombinant organisms has been reported in bacteria (*Genetic Engineering News*, Nov. 1991, p. 13): suicide genes were engineered to be expressed simultaneously with the desired recombinant gene product so that the recombinant bacteria die upon completion of their task. The present invention provides suicide genes which are useful in a variety of organisms in addition to bacteria, for example in insects, fungi, and transgenic rodents. Suicide genes can be constructed by placing the coding sequence of an exogenous cell death gene or an agonist of an endogenous cell death gene of the organism in an expression vector suitable for the organism.

In addition, agents which increase cell death are useful as pesticides (e.g., anthelmintics, nematocides). For example, many nematodes are human, animal, or plant parasites. *ced-3*, *ced-4*, or other nematode cell death genes, their gene products, mimetics, and agonists can be used to reduce the nematode population in an area, as well as to treat individuals already infected with the parasite or protect individuals from infection. A transgenic plant or animal carrying a constitutively activated *ced-3* gene, *ced-4* gene, or other cell death gene specific to

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The subject invention will now be illustrated by the following examples, which are not intended to be limiting in any way.²

MATERIALS AND METHODS

Techniques used for the culturing of *C. elegans* were essentially as described by Brenner (*Genetics* 77:71-94 (1974)). All strains were grown at 20°C. DNA was prepared from worms grown on Petri dishes
15 containing agarose seeded with *E. coli* strain HB101. RNA was prepared from mass cultures grown in liquid. Usually, the bacterial pellet from a 2 L overnight culture of *E. coli* HB101 grown in superbrot (12 g Bacto-tryptone, 24 g yeast extract, 8 ml 50% glycerol,
20 900 ml H₂O; after autoclaving, 100 ml 0.17 M KH₂PO₄ and 0.72 K₂HPO₄ were added) was resuspended in 500 ml S basal medium (Brenner, 1974 *supra*), and worms were added from one or two 10 cm Petri dishes in which the bacterial lawns had just been consumed. Worms were
25 harvested about 4-5 days later by centrifugation and washed in M9 buffer (Brenner, 1974 *supra*). The yield was about 5-10 ml of packed worms.

Nomarski differential interference contrast microscopy was used to examine individual cells in living ¹⁰nematodes (Sulston and Horvitz, *Dev. Biol.* 82:110-156 (1977)). Methods for scoring the Ced phenotype of *ced-1*, *ced-4* and *ced-1; ced-4* double

mutants have been described by Ellis and Horvitz, (*Cell* 44:817-829 (1986)) and by Yuan and Horvitz, (*Dev. Biol.* 138:33-41 (1990)).

The wild-type parent of all mutant strains used in these experiments was *C. elegans* variety Bristol strain N2 (Brenner, 1974 *supra*). The genetic markers used are listed below. These markers have been described (Brenner, 1974 *supra*; Hodgkin et al., in: *The Nematode Caenorhabditis elegans*, Wood and the Community of *C. elegans* Researchers (eds.), Cold Spring Harbor Laboratory, New York, 1988, pp. 491-584; Finney et al., *Cell* 55:757-769 (1988)). The strain TR679 carries the mutator *mut-2(r459)* (Collins et al., *Nature* 328:726-728 (1987)). The *ced-4* alleles *n1894*, *n1920*, *n1947*, *n1948*, *n2247*, and *n2273* were characterized in the present work. Genetic nomenclature follows the standard system for *C. elegans* (Horvitz et al., *Mol. Gen. Genet.* 175:129-133 (1979)):

- LG I: *ced-1(e1735)*, *unc-54(r323)*
- 20 LG III: *unc-86(n1351)*, *ced-4(n1162, n1416, n1894, n1920, n1947, n1948, n2247, n2273, n1416 n1712, n1416 n1713)*, *unc-79(e1068)*, *dpy-17(e164)*
- LG IV: *unc-31(e928)*, *ced-3(n717)*
- 25 LG V: *egl-1(n986)*, *unc-76(e911)*

Genomic Libraries

A 4-6 kb size-selected phage library was constructed from *ced-4(n1416)* DNA as follows. Genomic DNA was digested with *HindIII* and run on a low-melting agarose gel. DNA migrating within the 4-6 kb size range was excised, and the low-melting agarose was removed by phenol extraction and precipitation (Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1983)). These

DNA fragments were ligated to *Hind*III-digested DNA from phage λ NM1149 (Murray, *Phage Lambda and Molecular Cloning*, Cold Spring Harbor Laboratory, 1983, pp. 395-432). The product DNA was packaged with packaging
5 extract from Promega. This library had a total of 140,000 plaque-forming units (pfu), of which 70% were recombinants, as estimated from the ratio of pfu on bacteria C600hfl and C600.

The phage genomic library (provided by J. Sulston)
10 was prepared by partial digestion of wild-type *C. elegans* genomic DNA with *Sau*3A and cloning into the *Bam*HI site of phage vector λ 2001 (Karn et al., *Gene* 32:217-224 (1984)).

Tc4 Probe

15 The Tc4 probe used for cloning the *ced-4* gene and for Southern blots was Tc4-n1351, which contains a Tc4 element isolated from an *unc-86*(n1351) mutant strain (Finney et al., *Cell* 55:757-769 (1988); Yuan et al., *Proc. Natl. Acad. Sci. USA* 88:3334-3338 (1991)). DNA
20 was labelled with 32 P using either the nick-translation procedure described by Maniatis et al. (1983 *supra*) or the oligo-labelling procedure described by Feinberg and Vogelstein (*Anal. Biochem*, 132:6-13 (1983)).

RNA Preparation, Northern Blot and Primer Extension

25 Total *C. elegans* RNA was extracted using guanidine isothiocyanate (Kim and Horvitz, *Genes & Dev.* 4:357-371 (1990)). Poly(A)⁺ RNA was selected from total RNA by a poly(dT)-column (Maniatis et al., 1983 *supra*). To prepare stage-synchronized animals, eggs were obtained
30 from gravid *C. elegans* adults grown at 20°C in liquid culture. A 5 - 10 ml sample of animals was treated with 50 ml of NaOCl/NaOH solution (10 ml NaOCl, 1 g NaOH, 40 ml H₂O) for about 10 minutes with vortexing

The direction of transcription was determined by hybridizing northern blots with single-stranded RNA probes. The Bluescribe plasmid containing the insert pn1416 was linearized by digestion with either *Bam*HI or *Hind*III, which cleaved at one or the other end of the insert. The linearized product was transcribed using T3 or T7 RNA polymerase, respectively, generating RNA from each strand. These RNA products were used to probe Northern blots according to a protocol developed by Z. Liu and V. Ambros: Filters were prehybridized in 50% formamide, 50 mM sodium phosphate (pH 6.5), 5 x SSC, 8 X Denhardt's, 0.5% SDS, 250 µg/ml salmon sperm DNA and then hybridized with probe at 55°C and washed in 4 x SSC, 0.1% SDS at 60°C 3 times for 20 minutes each and then in 2 x SSC, 0.1% SDS once at 60°C for 20 minutes. Northern blot experiments showed that the single-stranded RNA probe transcribed by T3 RNA polymerase hybridized to the 2.2 kb *ced-4* mRNA, while the probe made by T7 RNA polymerase did not. This result indicates that the direction of the transcription is from the *Bam*HI site toward the *Hind*III site of pn1416.

25 For determining DNA sequences, serial deletions
were made according to Henikoff (Gene 28:351-359
(1984)). DNA sequences were determined using Sequenase
and protocols obtained from US Biochemicals (Cleveland,
OH). The *ced-4* DNA sequence was confirmed by
30 sequencing both strands of cDNA and genomic DNA clones.

The cosmid C10D8 was digested with *EcoRI*. Two *EcoRI* fragments of 2.2 kb (r5) and 2.4 kb (r7), both of

which hybridized to a mixture of *ced-4* cDNA subclones SK2-1 and SK2-2, were isolated. r7, which hybridized to SK2-1, which contains the 3' half of *ced-4* cDNA clone SK2, was cloned into the *EcoRI* site of plasmid pBSKII (Stratagene). The *EcoRI* site at the 3' end of r7 was deleted by digesting with *StyI*, which cut once at 0.2 kb from the 3' end of the insert, and *SalI*, which cut once in the polylinker, and then religating. The deleted r7 plasmid was linearized with *EcoRI* and ligated with *EcoRI*-digested r5, which hybridized to Sk2-2, the 5' half of *ced-4* cDNA SK2. Clones were analyzed for the correct orientation of the r5 insert based on the cDNA restriction map. One such correctly oriented clone was named C10D8-5.

15 Microinjection and Transformation

The procedure for microinjecting DNA into the gonad to obtain germline transformants was basically that of Fire (*EMBO J.* 5:2673-2680 (1986)) with modifications introduced by J. Sulston. Cosmid DNA to be injected was purified twice using CsCl-gradient centrifugation (Maniatis et al., 1983 *supra*). Plasmid DNA to be injected was prepared by alkaline minipreps (Maniatis et al., 1983 *supra*). DNA was treated with RNAase A (37°C, 30 minutes) and then with proteinase K (55°C, 30 minutes), extracted with phenol and then chloroform, precipitated twice (first in 0.3 M sodium acetate and then in 0.1 M potassium acetate, pH 7.2), and resuspended in 5 ul of injection buffer (Fire, 1986 *supra*). DNA concentrations used for injection were 0.1-1.0 mg/ml.

All transformation experiments used a *ced-1*; *ced-4(n1162)*; *unc-31* strain as the recipient. The expression of the *Ced-4* phenotype was quantified by counting the number of cell corpses in the heads of

young L1 animals. The cosmid C10D8 or plasmid subclones of C10D8 were mixed with cosmid C14G10, which contains the wild-type *unc-31(+)* gene, at a ratio of 2:1 or 3:1 to increase the likelihood that a phenotypically non-Unc transformant would contain the cosmid or plasmid being tested. Generally, 20-30 animals were injected in one experiment. Non-Unc F1 progeny of injected animals were isolated three to four days later. About 1/2 to 1/3 of the non-Unc progeny transmitted the non-Unc phenotype to their progeny and could be established as lines of transformants. Young L1 non-Unc progeny of such non-Unc transformants were examined using Nomarski optics to determine the number of cell corpses present in the heads.

15 Ced-4 Fusion Protein and Antibody Preparation

To express a Ced-4 fusion protein in *E. coli*, a clone containing both the 5' and 3' halves of the *ced-4* cDNA (SK2-2 and SK2-1) in the expression vector pET-5a (Rosenberg et al., *Gene* 56:125-135 (1987)) was constructed. The fusion protein expressed by this vector was expected to include 11 amino acids of phage T7 gene 10 protein, 5 amino acids of linker and the 546 amino acids encoded by *ced-4* cDNA SK2. The pJ76 plasmid, which encodes this fusion protein, was transformed into bacterial strain BL21. *ced-4* fusion protein was produced by this transformed strain, as expected, and subjected to electrophoresis on a polyacrylamide gel. A band, with mobility equivalent to about 64×10^3 Mr, specific to the transformed strain was exercised and used to immunize three rabbits. Sera from all three rabbits tested positive on western blots (Towbin et al., *Proc. Natl. Acad. Sci. USA* 76:4350-4354 (1979)). These sera were purified using immunoblots (Harlow and Lane, *Antibodies: A*

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[illegible][illegible][illegible][illegible]

To isolate additional genomic DNA from the region of this Tc4 insertion, pn1416 was used to probe a *C. elegans* Bristol N2 genomic DNA phage library. Five phage clones with inserts of 10 to

- 5 15 kb were isolated and shown to share a 3 kb *Bam*HI-*Hind*III fragment that hybridized to pn1416. These phage clones were used to identify cosmids that hybridized to them and that were members of a 600 kb contig of overlapping cosmids (Coulson et al., *Proc.*
10 *Natl. Acad. Sci. USA* 83:7821-7825 (1986)). By using the phage clones as probes to hybridize to Southern blots, a cosmid C10D8 was identified as containing all regions of genomic DNA present in all five phage clones and in pn1416.

15 The *ced-4* Mutant Phenotype Can Be Rescued by a 4.4 kb DNA Fragment

- To identify *ced-4*(+) DNA capable of complementing the *Ced-4* mutant phenotype, the cosmid C10D8 was injected into the oocytes of *ced-4*(n1162) animals. To
20 facilitate the identification of transgenic animals, a mutation in the *unc-31* gene, which affects locomotion, was included as a marker for co-transformation (Kim and Horvitz, *Genes & Dev.* 4:357-371 (1990)). Cosmid C14G10, which contains the wild-type allele of *unc-31*
25 and does not have *Ced-4*-rescuing activity was coinjected with cosmid C10D8 into *ced-1*(e1735); *unc-31*(e928); *ced-4*(n1162) animals. The *ced-1* mutation was included to facilitate the scoring of the *ced-4* mutant phenotype (Ellis and Horvitz, *Cell* 44:817-829
30 (1986)). Specifically, when a cell undergoes programmed cell death in *C. elegans*, its corpse is quickly engulfed and destroyed by a neighboring cell (Robertson and Thomson, *J. Embryol. Exp. Morph.* 67:89-100 (1982); Sulston et al., *Dev. Biol.* 100:64-119

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(1983)). A *ced-1* mutation prevents this engulfment, allowing the cell corpse to remain intact (Hedgecock et al., *Science* 220:1277-1280 (1983)). Thus, in a first or second stage (L1 or L2) *ced-1* mutant larva, many
5 cell corpses are present and can be easily visualized using Normaski optics. *ced-4* mutations prevent cell death and the appearance of these corpses. Thus, suppression of the *Ced-4* mutant phenotype by a wild-type *ced-4* gene can be observed and readily quantified
10 in a *ced-1* mutant background based on an increase in the number of visible cell corpses.

From one such microinjection experiment, three non-Unc animals rescued for the *Unc-31* mutant phenotype were picked from among the F1 progeny, and from one of
15 them a line of non-Unc transformants was obtained. No true-breeding non-Unc animals could be isolated from this line: about 25% of the progeny of all non-Unc animals were *Unc*. Since no inviable zygotes were observed among the progeny of these non-Unc animals,
20 this transformant did not carry a recessive lethal insertion mutation. Rather, it seems likely that the injected DNA was maintained as an extrachromosomal array that was segregated to only some gametes, as has been reported previously for many other *C. elegans*
25 transgenic strains (e.g., Stinchcomb et al., *Mol. Cell Biol.* 82:110-156 (1985); Fire, *EMBO J.* 5:2673-2680 (1986); Way and Chalfie, *Cell* 54:5-16 (1988)). This putative extrachromosomal array was named *nEx1*. Young L1 progeny of *nEx1*-containing animals were examined
30 using Nomarski optics for the *Ced-4* phenotype.

Young L1 *ced-1* animals have an average of 23 cell corpses in the head, while *ced-1(e1735); ced-4(n1162)* animals have an average of 0.6 cell corpses (Ellis and Horvitz, *Cell* 44:817-829 (1986)). Young L1 *ced-1*;
35 *ced-4(n1162); nEx1* animals had an average of nine cell

corpses in the head. These results indicate that cosmid C10D8 restored significant, but not total, *ced-4(+)* activity in the transformants.

To delineate the *ced-4* gene within C10D8, various subclones of C10D8 were injected into *ced-4* mutant animals and tested for their ability to rescue the *Ced-4* mutant phenotype (Table 1). The smallest subclone plasmid that could rescue the *ced-4* phenotype as effectively as cosmid C10D8 was a 4.4 kb fragment, called C10D8-5. C10D8-5 and the *unc-31(+)*-containing cosmid C14G10 were coinjected into *ced-1; unc-31; ced-4(n1162)* animals. Two lines of non-Unc transformants were isolated. Since these animals continued to segregate Unc animals and did not produce inviable zygotes, both appeared to carry extrachromosomal arrays, which were designated *nEx7* and *nEx8*. Young L1 animals from these transformant strains had an average of 11.5 cell corpses in their heads, indicating that plasmid C10D8-5 restored *ced-4(+)* activity as well as did cosmid C10D8 (Table 1).

Identification of a *ced-4* Transcript

Restriction sites of plasmid C10D8-5 (which can rescue the *Ced-4* phenotype) and pn1416 (which contains sequences adjacent to the Tc4 insertion site) were mapped. C10D805 was found to overlap with 2 kb of sequence in pn1416, including the Tc4 insertion site (Figure 8).

In Northern blot experiments, both pn1416 and C10D8-5 were used to probe poly(A)⁺ RNA populations of mixed developmental stages of wild-type (strain N2), *ced-4(n1416)*, and *ced-4(n1416 n1712)* and *ced-4(n1416 n1713)*⁷⁷¹ revertant animals. pn1416 hybridized to a 2.2 kb transcript and an 0.9 kb transcript in RNA from N2 animals, and a 3 kb transcript, a transcript slightly

To determine if any of the transcripts contains Tc4 sequence, the Northern blots were probed with Tc4-n1351, which contains the 1.6 kb Tc4 element present in the Tc4-induced mutant *unc-86(n1351)* as well as 4 kb of *unc-86* sequences. Tc4-n1351 hybridized both to a 3.8 kb transcript of the Tc4-induced mutant *ced-4(n1416)* and to a 1.5 kb *unc-68* transcript in both *ced-4(n1416)* and N2 animals.

To determine whether one or both of the 2.2 kb and
35 0.9 kb transcripts are encoded by *ced-4*, subclone

C10D8-5, which rescued the *Ced-4* phenotype, was used to probe the Northern blots. C10D8-5 detected the wild-type 2.2 kb transcript, the *ced-4(n1416)* transcript slightly larger than the 2.2 kb transcript, and the *ced-4(n1416)* 3.8 kb transcript. C10D8-5 did not hybridize to the 0.9 kb transcript, indicating that this transcript is unlikely to be encoded by *ced-4*. C10D8-5 also detected a 1.4 kb transcript, which was not altered by the Tc4 insertion in *ced-4(n1416)* animals. Only a 470 bp *EcoRI-StuI* fragment at one end of C10D8-5 hybridized to this 1.4 kb RNA. Since C10D8-5 did not contain the complete coding region for this RNA, and since this RNA was unaffected in *ced-4(n1416)* animals, this 1.4 kb RNA seems unlikely to be a *ced-4* transcript. The relationships among cosmid C10D8-5, pn1416 and the 0.9 kb, 1.4 kb and 2.2 kb transcripts are summarized in Figure 8.

On Northern blots probed with the *ced-4* cDNA clone SK2-1, the level of the 2.2 kb transcript showed significant reduction in all three independently derived EMS-induced *ced-4* mutants examined, strongly supporting the hypothesis that this 2.2 kb transcript is a *ced-4* transcript. Total RNA from N2, *ced-4(n1162)*, *ced-4(n1416)*, *ced-4(n1894)* and *ced-4(n1920)* eggs was probed with ³²P-labelled *ced-4* cDNA SK2-1. An actin 1 probe (Krause and Hirsh, in: *Molecular Biology of the Cytoskeleton*, Borisy et al. (eds.), Cold Spring Harbor Laboratory, 1984, pp. 287-292) was used as an internal control for the amount of RNA loaded in each lane. The ratios of the intensity of the *ced-4* band to that of actin band in N2, n1162, n1416 and n1894 were 0.5, 0.17, 0 and 0.12, respectively. A Northern blot of poly(A)⁺ RNA from stage-synchronized animals was probed with pn1416, which hybridizes both to the 2.2 kb *ced-4* transcript

and to a 0.9 kb transcript. The 0.9 kb transcript seems to be expressed mostly in eggs and adults. The presence of RNA in all lanes was confirmed by loading 1/10 of each sample on another gel and probing a Northern blot from this gel using the *C. elegans* actin 1 gene (Krause and Hirsh, 1984 *supra*). That all of these distinct *ced-4* mutations cause reduced levels of a *ced-4* transcript could reflect either instability of all three mutant transcripts or a role for *ced-4* in regulating its own expression.

Based upon these results, it can be concluded that the 2.2 kb RNA is a *ced-4* transcript. It is not known why the 0.9 kb RNA is also altered in *ced-4(n1416)* animals. Perhaps transcription of the 0.9 kb RNA is initiated incorrectly as a consequence of the nearby Tc4 element.

ced-4 Expression is Primarily Embryonic

A Northern blot containing RNAs from stage-synchronized animals of different developmental stages probed with pn1416 showed that the 2.2 kb *ced-4* transcript was expressed primarily during embryogenesis. This result is consistent with the observation that 113 of the 131 programmed cell deaths in the *C. elegans* hermaphrodite are embryonic (Sulston and Horvitz, *Dev. Biol.* 82:110-156 (1977); Sulston et al., *Dev. Biol.* 100:64-119 (1983)). The 2.2 kb RNA was relatively abundant during embryonic development. The 0.9 kb transcript was expressed mostly in eggs and adults. The presence of RNA in all lanes was confirmed by loading 1/10 of each sample on another gel and probing a Northern blot from this gel with the *C. elegans* actin 1 gene (Krause and Hirsh, 1984 *supra*).

The *ced-4* Transcript is Present in a *ced-3* Mutant

The activities of both *ced-3* and *ced-4* are required for programmed cell death (Ellis and Horvitz, Cell 44:817-819 (1986)). One possibility is that one of these genes positively regulates the expression of the other. For this reason, a Northern blot of wild-type strain N2 and *ced-3*(n717) poly(A)⁺ RNA was probed with pn1416. This experiment showed that the 2.2 kb *ced-4* transcript was present at an apparently normal level in this *ced-3* mutant. Thus, the activity of the *ced-3* gene is unlikely to be necessary for the expression of the *ced-4* 2.2 kb transcript.

Identification of *ced-4* cDNA Clones

To isolate cDNA clones of *ced-4*, pn1416 was used to probe a *C. elegans* cDNA phage library made from wild-type strain N2 mixed-stage RNA (Kim and Horvitz, Genes & Dev. 4:357-371 (1990)). Two cDNA clones were isolated. The two cDNA clones (named SK1 and SK2) hybridized to the 2.2 kb *ced-4* transcript. Both are about 1.8 kb in size, and both contain one 0.8 kb and one 1.0 kb *EcoRI* fragment. These *EcoRI* fragments were subcloned into plasmid vector Bluescribe M13+ (Stratagene). The two subclones derived from SK1 were named SK1-1 and SK1-2, and the two subclones derived from SK2 were named SK2-1 and SK2-2. The restriction maps of the SK1- and SK2-derived clones were the same. Sequence analysis of the ends of the four cDNA subclones confirmed the equivalence of the SK1 and SK2 clones, except that SK1-2 contains a poly(A) sequence of more than 50 bp at its 5' end. This poly(A) sequence is probably a cDNA cloning artifact, since SK1-2 contains the 5' half of the cDNA (see below).

The *ced-4* Sequence

The DNA sequence of the SK2 1.8 kb cDNA clone was determined. This sequence includes an open reading frame encoding 546 amino acids (Figure 1; Seq. ID #2), which is consistent with the results of Northern blot analysis using single-stranded RNA probes. An ochre termination codon (TAA) is located in-frame near the 3' end, indicating that the 3' end of the 2.2 kb transcript is most likely included in this cDNA. The open reading frame extends to the 5' end of the 1.8 kb cDNA, suggesting that this cDNA might lack the 5' end of the *ced-4* coding region.

A primer extension experiment was performed to determine the *ced-4* transcription initiation site(s) using the primer ATTGGCGATCCTCTCGA (Seq. ID #23) and C10D8-5 as template. A major transcriptional initiation site was identified at 54 bp before (5' of) the beginning of the *ced-4* cDNA SK2 and a minor initiation site at 54 bp after (3' of) the beginning of this cDNA (Figure 1). The first AUG codon after the presumptive major start site is located at 9 bp before the beginning of the cDNA (Figure 1). If this site is used to initiate protein synthesis, the Ced-4 protein would be 549 amino acids in length. The first AUG codon after the presumptive minor start site is located at 130 bp after the beginning of the cDNA. If this site is used, the Ced-4 protein would be 503 amino acids in length. Preliminary results using an anti-Ced-4 antibody raised against a Ced-4 fusion protein showed that endogenous Ced-4 protein is slightly smaller in molecular weight than a Ced-4 fusion protein of 562 amino acids expressed in *E. coli*. Thus, most Ced-4 protein is probably initiated near the start of the cDNA and is presumably 549 amino acids in length and 62,977 in relative molecular mass. The direction

of the open reading frame is consistent with the direction of transcription, as demonstrated by probing Northern blots with single-stranded RNA probes. The presumptive Ced-4 protein is highly hydrophilic, with a pI of 5.12. The longest hydrophobic region is a segment of 12 amino acids from residues 382 to 393.

A Western blot of wild-type strain N2 mixed-stage, *ced-4(n1416)* mixed-stage, wild-type egg, and bacterially expressed protein (pJ76) was probed using anti-Ced-4 antibody. Ced-4 fusion protein (pJ76) was made by cloning *ced-4* cDNA SK2 into the T7 expression vector pET-5a (Rosenberg et al., Gene 56:125-135 (1987)), so that 546 amino acids of Ced-4 sequence were fused to 11 amino acids of T7 gene 10 protein and 5 amino acids of linker sequence. This Ced-4 fusion protein is similar in relative molecular mass to the endogenous Ced-4 protein, which is present in wild-type (N2) but missing in *ced-4(n1416)* animals. The proteins phosphorylase b, 97×10^3 ; bovine serum albumin, 66×10^3 (Hirayama et al., Biochem. Biophys. Res. Comm. 173:639-646 (1990)); and ovalbumin, 43×10^3 , were used as molecular weight standards.

To confirm the DNA sequence obtained from the *ced-4* cDNAs and to study the structure of the *ced-4* gene, the sequences of the 4.4 kb cosmid subclone C10D8-5, the 3 kb insert pn1416, and the 2 kb *HindIII*-*BamHI* fragment that contains the Tc4 insertion in the *ced-4(n1416)* mutant were determined. Comparison of the *ced-4* genomic and cDNA sequences revealed that the *ced-4* gene has seven introns of sizes ranging from 44 bp to 557 bp (Figure 2). The exon sequences of genomic clone C10D8-5 are identical to the sequences of *ced-4* cDNA SK2. Comparison of the Tc4 insertion site in *ced-4(n1416)* DNA with the *ced-4(+)* genomic and cDNA

sequences indicated that Tc4 inserted into an exon in the *ced-4* gene in *ced-4(n1416)* animals (Figure 2).

The DNA sequences of eight EMS-induced *ced-4* alleles were also determined (Table 2). One of the eight, *n1948*, is a missense mutation. Of the seven others, four create stop codons and three are predicted to affect splicing of the *ced-4* transcript. The positions of these mutations are indicated in Figure 2. These findings indicate that the phenotypes of these mutants (Ellis and Horvitz, *Cell* 44:817-829 (1986)) result from a complete loss of *ced-4* gene function. These mutations establish the null phenotype of the *ced-4* gene, confirming that *ced-4* function is not essential for viability.

15 The Ced-4 Protein Has Two Regions Similar to Known Calcium-Binding Domains

By direct inspection, the sequence of the putative Ced-4 protein was compared with the consensus sequence of the calcium-binding loop of the EF-hand domain (Tufty and Kretsinger, *Science* 187:161-171 (1975); Kretsinger, *Cold Spring Harbor Symp. Quant. Biol.* 52:499-510 (1987); Szebenyi and Moffat, *J. Biol. Chem.* 26:8761-8777 (1986)). Two regions of the Ced-4 protein were identified that might bind calcium (Figure 3).

25 The EF-hand is a 29 amino acid domain consisting of a helix-loop-helix region, with the loop portion (residues 10-21) coordinating calcium-binding via the side-chain oxygens of serine, threonine, asparagine, aspartic acid, glutamine or glutamic acid. These residues occur at five of the vertices of an octahedron: X (position 10), Y (12), Z (14), -X (18), -Z (21). EF-hand amino acid sequences vary considerably in the residues present in the calcium-binding loop (Figure 3), and some EF-hand domains have

Positions Y, Z, and -X can have any of a number of amino acids which have oxygen-containing side chains.

The sequences of parvalbumins from carp muscle (Seq. ID #3; Nockolds et al., *Proc. Natl. Acad. Sci. USA* 69:581-584 (1972)), the intestinal calcium-binding protein (ICaBP) (Seq. ID #7-8; Szebenyi et al., *Nature* 294:327-332 (1981)), troponin C (Seq. ID #9-12; Collins et al., *FEBS Lett.* 36:268-272 (1973)) and calmodulin (Seq. ID #13; Zimmer et al., *J. Biol. Chem.* 263:19,370-19,383 (1988); Babu et al., *Nature* 315:37-40 (1985)) show canonical EF-hands. The hake and ray parvalbumins (Seq. ID #4-5; Capony et al. *Eur. J. Biochem.* 32:97-108 (1973)); Thatcher and Pechere, *Eur. J. Biochem.* 75:121-132 (1977)), sarcoplasmic calcium-binding protein (SCBP) from the protochordate *Amphioxus* (Seq. ID #6; Takagi et al., *Biochemistry* 25: 3585-3592 (1986)), trypsinogen (Seq. ID #14; Bode and Schwager, *J. Mol. Biol.* 98:693-717 (1975)), fibrinogen (Seq. ID #15; Doolittle, *Ann. Rev. Biochem.* 53:195-229 (1984); Dang et al., *J. Biol. Chem.* 260:9713-9719 (1985)), villin (Seq. ID #16; Hesterberg and Weber, *J. Biol. Chem.* 258:365-369 (1983)) and galactose-binding protein (GBP) (Seq. ID #17; Vyas et al., *Nature* 327:635-638 (1987)) show variations from the consensus sequence. GBP does not contain the helices of the EF-hand.

30 The potential calcium-binding loops of sequence 1
and sequence 2 are located at amino acids 77-88 and
amino acids 292-303 of the Ced-4 protein, respectively
(Figure 3). In its putative calcium-binding loop, the
first potential EF-hand-like sequence of the Ced-4
35 protein has four (positions Y, Z, -X, -Z) of the five

followed by alpha-helical domains (positions 22-29)
(Kretsinger, 1987 *supra*). Since position 3 of Ced-4
sequence 1 and positions 26 and 28 of Ced-4 sequence 2
are prolines, these regions might not form alpha-
5 helices. However, the known calcium-binding protein
galactose-binding protein (GBP) has a calcium-binding
domain similar to that of the EF-hand (Figure 3) but
without the two helices; furthermore, position 29 of
GBP is proline (Vyas et al., 1987 *supra*). Thus, the
10 Ced-4 protein need not contain such alpha-helical
calcium-binding domains.

Based upon these considerations, it seems likely
that the Ced-4 protein binds calcium or a similar
divalent cation.

15

EXAMPLE 2

CLONING, SEQUENCING, AND CHARACTERIZATION OF THE CED-3 GENE

MATERIALS AND METHODS

General Methods and Strains

20 The techniques used for the culturing of *C.*
elegans were as described by Brenner (*Genetics* 77:71-94
(1974)). All strains were grown at 20°C. The wild-
type parent strains were *C. elegans* variety Bristol
strain N2, Bergerac strain EM1002 (Emmons et al., *Cell*
25 32:55-65 (1983)), *C. briggsae* and *C. vulgaris* (obtained
from V. Ambros). The genetic markers used are
described below. These markers have been described by
Brenner (1974 *supra*), and Hodgkin et al. (In: *The*
Nematode Caenorhabditis elegans, Wood and the Community
30 of *C. elegans* Researchers (eds.), Cold Spring Harbor
Laboratory, 1988, pp 491-584). Genetic nomenclature

follows the standard system (Horvitz et al., Mol. Gen. Genet. 175:129-133 (1979)).

- LG I: *ced-1(e1375); unc-54(r323)*
LG VI: *unc-31(e928), unc-30(e191), ced-3(n717, n718,*
5 *n1040, n1129, n1163, n1164, n1165, n1286,*
n1949, n2426, n2430, n2433), unc-26(e205),
dpy-4(e1166)
LG V: *egl-1(n986); unc-76(e911)*
LG X: *dpy-3(e27)*

10 Isolation of Additional Alleles of *ced-3*

A non-complementation screen was designed to isolate new alleles of *ced-3*. Because animals heterozygous for *ced-3(n717)* in trans to a deficiency are viable (Ellis and Horvitz, Cell 44:817-829 (1986)),
15 animals carrying a complete loss-of-function *ced-3* allele generated by mutagenesis were expected to be viable in trans to *ced-3(n717)*, even if the new allele was inviable in homozygotes. Fourteen EMS mutagenized *egl-1* males were mated with *ced-3(n717) unc-26(e205);*
20 *egl-1(n487); dpy-3(e27)* hermaphrodites. *egl-1* was used as a marker in this screen. Dominant mutations in *egl-1* cause the two hermaphrodite specific neurons, the HSNs, to undergo programmed cell death (Trent et al., Genetics 104:619-647 (1983)). The HSNs are required
25 for normal egg-laying, and *egl-1(n986)* hermaphrodites, which lack HSNs, are egg-laying defective (Trent et al., 1983 supra). The mutant phenotype of *egl-1* is suppressed in a *ced-3; egl-1* strain because mutations in *ced-3* block programmed cell deaths. *egl-1* males
30 were mutagenized with EMS and crossed with *ced-3(n717), unc-26(e205); egl-1(n487); dpy-3(e27)*. Most cross progeny were egg-laying defective because they were heterozygous for *ced-3* and homozygous for *egl-1*. Rare

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Molecular Biology

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The "right" end of MMM-C1 was cloned by cutting it with HindIII and self-ligating. The "left" end of MMM-C1 was cloned by cutting it with *Bgl*II or *Sal*I and self-ligating.

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The Tc1 DNA probe for Southern blots was pCe2001, which contains a Bergerac Tc1 element (Emmons et al., Cell 32:55-65 (1983)). Enzymes were purchased from New England Biolabs, and radioactive nucleotides were from Amersham.

Primer extension procedures followed the protocol by Robert E. Kingston (In: *Current Protocols in Molecular Biology*, Ausubel et al. (eds.), Greene Publishing Associates and Wiley-Interscience, New York, p. 4.8.1) with minor modifications.

Polymerase chain reaction (PCR) was carried out using standard protocols supplied by the GeneAmp Kit (Perkin Elmer). The primers used for primer extension and PCR are as follows:

15 Pex2: 5' TCATCGACTTTTAGATGACTAGAGAACATC 3'
(Seq. ID #24);
Pex1: 5' GTTGCACTGCTTTCACGATCTCCCGTCTCT 3'
(Seq. ID #25);
SL1: 5' GTTTAATTACCCAAGTTTGAG 3' (Seq. ID #26);
20 SL2: 5' GGTTTAAACCACTTACTCAAG 3' (Seq. ID #27);
Log5: 5' CCGGTGACATTGGACACTC 3' (Seq. ID #28); and
Oligo10: 5' ACTATTCAACACTTG 3' (Seq. ID #29).

Germline Transformation

The procedure for microinjection basically follows that of A. Fire (EMBO J. 5:2673-2680 (1986)) with modifications: Cosmid DNA was twice purified by CsCl-gradient. Miniprep DNA was used when deleted cosmids were injected. To prepare miniprep DNA, DNA from 1.5 ml overnight bacterial culture in superbrot (12 g Bacto-tryptone, 24 g yeast extract, 8 ml 50% glycerol, 900 ml H₂O, autoclaved; after autoclaving, 100 ml 0.17 M KH₂PO₄ and 0.72 M KH₂PO₄ were added) was extracted by alkaline lysis method as described in Maniatis et al.

(1983 *supra*). DNA was treated with RNase A (37°, 30 minutes) and then with protease K (55°, 30 minutes), extracted with phenol and then chloroform, precipitated twice (first in 0.3 M sodium acetate and second in 0.1 M potassium acetate, pH 7.2), and resuspended in 5 μ l injection buffer as described by A. Fire (1986 *supra*). The DNA concentration for injection is in the range of 100 ug to 1 mg per ml.

All transformation experiments used *ced-1(e1735); unc-31(e928) ced-3(n717)* strain. *unc-31* was used as a marker for co-transformation (Kim and Horvitz, 1990 *supra*). *ced-1* was present to facilitate scoring of the *ced-3* phenotype. The mutations in *ced-1* block the engulfment process of cell death, which makes the corpses of the dead cells persist much longer than in wild-type animals (Hedgecock et al., *Science* 220:1277-1280 (1983)). The *ced-3* phenotype was scored as the number of dead cells present in the head of young L1 animals. The cosmid C10D8 or the plasmid subclones of C10D8 were mixed with C14G10 (*unc-31(+)*-containing) at a ratio of 2:1 or 3:1 to increase the chances that a *Unc-31(+)* transformant would contain the cosmid or plasmid being tested as well. Usually, 20-30 animals were injected in one experiment. Non-Unc F1 progeny of the injected animal were isolated three to four days later. About 1/2 to 1/3 of the non-Unc progeny transmitted the non-Unc phenotype to F2 progeny and established a transformant line. The young L1 progeny of such non-Unc transformant were checked for the number of dead cells present in the head using Nomarski optics.

Isolation of Additional *ced-3* Alleles

Mapping RFLPs near ced-3

20 Tc1 is a *C. elegans* transposable element that is thought to be immobile in the common laboratory Bristol strain and in the Bergerac strain (Emmons et al., Cell 32:55-65 (1983)). In the Bristol strain, there are 30 copies of Tc1, while in the Bergerac strain, there are
25 more than 400 copies of Tc1 (Emmons et al., 1983 *supra*; Finney, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, Massachusetts, 1987). Because the size of the *C. elegans* genome is small (haploid genome size 8×10^7 bp) (Sulston and Brenner, Genetics
30 77:95-104 (1976)), a polymorphism due to Tc1 between the Bristol and Bergerac strains would be expected to occur about once every 200 kb. Restriction fragment

length polymorphisms (RFLPs) can be used as genetic markers and mapped in a manner identical to conventional mutant phenotypes. A general scheme has been designed to map Tc1 elements that are dimorphic
5 between the Bristol and Bergerac strains near any gene of interest (Ruvkun et al., *Genetics*, 121:501-516 (1989)). Once tight linkage of a particular Tc1 to a gene of interest has been established, that Tc1 can be cloned and used to initiate chromosome walking.

10 A 5.1 kb Bristol-specific Tc1 *EcoRI* fragment was tentatively identified as containing the Tc1 closest to *ced-3*. This Tc1 fragment was cloned using cosmids from a set of Tc1-containing *C. elegans* Bristol genomic DNA fragments. DNA was prepared from 46 such Tc1-
15 containing cosmids, and this DNA was screened using Southern blots to identify the cosmids that contain a 5.1 kb *EcoRI* Tc1-containing fragment. Two such cosmids were identified: MMM-C1 and MMM-C9. The 5.1 kb *EcoRI* fragment was subcloned from MMM-C1 into pUC13
20 (Promega). Since both ends of Tc1 contain an *EcoRV* site (Rosenzweig et al., *Nucleic Acids Res.* 11:4201-4209 (1983)), *EcoRV* was used to remove Tc1 from the 5.1 kb *EcoRI* fragment, generating a plasmid that contains only the unique flanking region of this Tc1-containing
25 fragment. This plasmid was then used to map the specific Tc1 without the interference of other Tc1 elements.

unc-30(e191) ced-3(n717) dpy-4(e1166)/+++ males were crossed with Bergerac (EM1002) hermaphrodites, and
30 Unc non-Dpy or Dpy non-Unc recombinants were picked from among the F2 progeny. The recombinants were allowed to self-fertilize, and strains that were homozygous for either *unc-30(e191) dpy-4(Bergerac)* or *unc-30(Bergerac) dpy-4(e1166)* were isolated. After
35 identifying the *ced* genotypes of these recombinant

strains, DNA was prepared from these strains. A Southern blot of DNA from these recombinants was probed with the flanking sequence of the 5.1 kb *EcoRI* *Tc1* fragment. This probe detects a 5.1 kb fragment in Bristol N2 and a 3.4 kb fragment in Bergerac. Five out of five *unc-30 ced-3 dpy(+Berg)* recombinants, and one of one *unc-30(+Berg) ced-3 dpy-4* recombinants showed the Bristol pattern. Nine of ten *unc-30(+Berg) dpy-4* recombinants showed the Bergerac pattern. Only one recombinant of *unc-30(+Berg) dpy-4* resulted from a cross-over between *ced-3* and the 5.1 kb *Tc1* element. The genetic distance between *ced-3* and *dpy-4* is 2 map units (mu). Thus, this *Tc1* element is located 0.1 mu on the right side of *ced-3*.

Cosmids MMM-C1 and MMM-C9 were used to test whether any previously mapped genomic DNA cosmids overlapped with these two cosmids. A contig of overlapping cosmids was identified that extended the cloned region near *ced-3* in one direction.

To orient MMM-C1 with respect to this contig, both ends of MMM-C1 were subcloned and these subclones were used to probe the nearest neighboring cosmid C48D1. The "right" end of MMM-C1 does not hybridize to C48D1, while the "left" end does. Therefore, the "right" end of MMM-C1 extends further away from the contig. To extend this contig, the "right" end of MMM-C1 was used to probe the filters of two cosmid libraries (Coulson et al., *Proc. Natl. Acad. Sci. USA* 83:7821-7825 (1986)). One clone, Jc8, was found to extend MMM-C1 in the opposite direction of the contig.

RFLPs between the Bergerac and Bristol strains were used to orient the contig with respect to the genetic map. Bristol (N2) and Bergerac (EM1002) DNA was digested with various restriction enzymes and probed with different cosmids to look for RFLPs. Once

such an RFLP was found, DNA from recombinants of the Bristol and Bergerac strains between *ced-3* and *unc-26*, and between *unc-30* and *ced-3* was used to determine the position of the RFLP with respect to *ced-3*.

5 The "right" end of Jc8, which represents one end of the contig, detects an RFLP (*nP33*) when N2 and EM1002 DNA was digested with *HindIII*. A Southern blot of DNA from recombinants between three *ced-3*(+Berg) *unc-26* was probed with the "right" end of Jc8. Three
10 of three +Berg *unc-26* recombinants showed the Bristol pattern, while two of two *ced-3 unc-26*(+Berg) recombinants showed the Bergerac pattern. Thus, *nP33* mapped very close or to the right side of *unc-26*.

26E02T BCT48680
15 The "left" end of Jc8 also detects a *HindIII* RFLP (*nP34*). The same Southern blot was reprobed with the Jc8 "left" end. Two of the two *ced-3 unc-26*(+Berg) recombinants and two of the three *ced-3*(+Berg) *unc-26* recombinants showed the Bergerac pattern. One of the three *ced-3*(+Berg) *unc-26* recombinants showed the
20 Bristol pattern. The genetic distance between *ced-3* and *unc-26* is 0.2 mu. Thus, *nP34* was mapped between *ced-3* and *unc-26*, about 0.1 mu on the right side of *ced-3*.

25 The flanking sequence of the 5.1 kb *EcoRI* Tc1 fragment (named *nP35*) was used to probe the same set of recombinants. Two of three *ced-3*(+Berg) *unc-26* recombinants and two of two *ced-3 unc-26*(+Berg) recombinants showed the Bristol pattern. Thus, *nP35* was also found to be located between *ced-3* and *unc-26*,
30 about 0.1 mu on the right side of *ced-3*.

A similar analysis using cosmid T10H5 which contains the *HindIII* RFLP (*nP36*), and cosmid B0564, which contains a *HindIII* RFLP (*nP37*), showed that *nP36* and *nP37* mapped very close or to the right of *unc-30*.

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Complementation of *ced-3* by Germline Transformation

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As shown in Table 4, of the three cosmids injected (C43C9, W07H6 and C48D1), only C48D1 rescued the *ced-3*

phenotype (2/2 non-Unc transformants rescued the *ced-3* phenotype). One of the transformants, *nEX2*, appears to be rescued by an extra-chromosomal array of injected cosmids (Way and Chalfie, Cell 54:5-16 (1988)), which is maintained as an unstable duplication, since only 50% of the progeny of a non-Unc *Ced(+)* animal are non-Unc *Ced(+)*. Since the non-Unc *Ced(+)* phenotype of the other transformant (*nIS1*) is transmitted to all of its progeny, it is presumably an integrated transformant. L1 *ced-1* animals contain an average of 23 cell corpses in the head (Table 5). L1 *ced-1; ced-3* animals contain an average of 0.3 cell corpses in the head. *ced-1; unc-31 ced-3; nIS1* and *ced-1; unc-31 ced-3; nEX2* animals contain an average of 16.4 and 14.5 cell corpses in the head, respectively. From these results, it was concluded that C48D1 contains the *ced-3* gene.

In order to locate *ced-3* more precisely within the cosmid C48D1, this cosmid was subcloned and the subclones were tested for the ability to rescue *ced-3* mutants (Table 5). C48D1 DNA was digested with restriction enzymes that cut rarely within the cosmid and the remaining cosmid was self-ligated to generate a subclone. Such subclones were then injected into a *ced-3* mutant to look for complementation; young L1 non-Unc progeny of the transformants were examined using Nomarski optics for the presence of cell death in the head. When C48D1 was digested with *Bam*HI and self-ligated, the remaining 14 kb subclone (named C48D1-28) was found to rescue the *ced-3* phenotype when injected into a *ced-3* mutant (Figure 10 and Table 5). C48D1-28 was then partially digested with *Bgl*III and self-ligated. Clones of various lengths were isolated and tested for their ability to rescue *ced-3*.

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One clone, C48D1-43, which did not contain a 1.7 kb *Bgl*III fragment of C48D1-28, was able to rescue *ced-3* (Figure 10 and Table 5). C48D1-43 was further subcloned by digesting with *Bam*HI and *Apa*I to isolate a 10 kb *Bam*HI-*Apa*I fragment. This fragment was subcloned into pBSKII+ to generate pJ40. pJ40 can restore *ced-3*+ phenotype when microinjected into a *ced-3* mutant. pJ40 was subcloned by deleting a 2 kb *Bgl*III-*Apa*I fragment to generate pJ107. pJ107 was also able to rescue the *ced-3* phenotype when microinjected into a *ced-3* mutant. Deletion of 0.5 kb on the left side of pJ107 could be made by *Exo*III digestion (as in pJ107del28 and pJ107del34) without affecting *ced-3* activity; in fact, one transgenic line, *nEX17*, restores full *ced-3* activity. However, the *ced-3* rescuing ability was significantly reduced when 1 kb was deleted on the left side of pJ107 (as in pJ107del12 and pJ107del27), and the ability was completely eliminated when a 1.8 kb *Sal*I-*Bgl*III fragment was deleted on the right side of pJ107 (as in pJ55 and pJ56), suggesting that this *Sal*I site is likely to be in the *ced-3* coding region. From these experiments, *ced-3* was localized to a DNA fragment of 7.5 kb. These results are summarized in Figure 10 and Table 5.

25 *ced-3* Transcript

pJ107 was used to probe a Northern blot of N2 RNA and detected a band of 2.8 kb. Although this transcript is present in 12 *ced-3* mutant animals, subsequent analysis showed that all 12 *ced-3* mutant alleles contain mutations in the genomic DNA that codes for this mRNA (see below), thus establishing this RNA as a *ced-3* transcript.

The developmental expression pattern of *ced-3* was determined by hybridizing a Northern blot of RNA from

animals of different stages (eggs, L1 through L4 larvae and young adult) with the *ced-3* cDNA subclone pJ118. Such analysis revealed that the *ced-3* transcript is most abundant during embryonic development, which is the period when most programmed cell deaths occur, but it was also detected during the L1 through L4 larval stages and is present in relatively high levels in young adults. This result suggests that *ced-3* is not only expressed in cells undergoing programmed cell death.

Since *ced-3* and *ced-4* are both required for programmed cell death in *C. elegans*, one of the genes might act as a regulator of transcription of the other gene. To examine if *ced-4* regulates the transcription of *ced-3*, RNA was prepared from eggs of *ced-4* mutants (*n1162*, *n1416*, *n1894*, and *n1920*), and a Northern blot was probed with the *ced-3* cDNA subclone pJ118. The presence of RNA in each lane was confirmed with an actin I probe. Such an experiment showed that the level of *ced-3* transcript is normal in *ced-4* mutants. This indicates that *ced-4* is unlikely to be a transcriptional regulator of *ced-3*.

Isolation of a *ced-3* cDNA

To isolate cDNA of *ced-3*, pJ40 was used as a probe to screen a cDNA library of N2 (Kim and Horvitz, *Genes & Dev.* 4:357-371 (1990)). Seven cDNA clones were isolated. These cDNAs can be divided into two groups: one is 3.5 kb and the other 2.5 kb. One cDNA from each group was subcloned and analyzed further. pJ85 contains the 3.5 kb cDNA. Experiments showed that pJ85 contains a *ced-3* cDNA fused to an unrelated cDNA; on Northern blots of N2 RNA, the pJ85 insert hybridizes to two RNA transcripts, and on Southern blots of N2 DNA, pJ85 hybridizes to more than one band than pJ40 (*ced-3*

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genomic DNA) does. pJ87 contains the 2.5 kb cDNA. On Northern blots, pJ87 hybridizes to a 2.8 kb RNA and on Southern blots, it hybridizes only to bands to which pJ40 hybridizes. Thus, pJ87 contains only *ced-3* cDNA.

5 To show that pJ87 does contain the *ced-3* cDNA, a frameshift mutation was made in the *SalI* site of pJ40 corresponding to the *SalI* site in the pJ87 cDNA. Constructs containing the frameshift mutation failed to rescue the *ced-3* phenotype when microinjected into
10 *ced-3* mutant animals, suggesting that *ced-3* activity has been eliminated.

ced-3 Sequence

The DNA sequence of pJ87 was determined (see Figure 4; Seq. ID #18). pJ87 contains an insert of 2.5
15 kb which has an open reading frame of 503 amino acids (Figure 4; Seq. ID #19). The 5' end of the cDNA contains 25 bp of poly-A/T sequence, which is probably an artifact of cloning and is not present in the genomic sequence. The cDNA ends with a poly-A
20 sequence, suggesting that it contains the complete 3' end of the transcript. 1 kb of pJ87 insert is untranslated 3' region and not all of it is essential for *ced-3* expression, since genomic constructs with deletions of 380 bp of the 3' end can still rescue
25 *ced-3* mutants (pJ107 and its derivatives, see Figure 10).

To confirm the DNA sequence obtained from the *ced-3* cDNA and to study the structure of the *ced-3* gene, the genomic sequence of the *ced-3* gene in the
30 plasmid pJ107 was determined (Figure 4; Seq. ID #18). Comparison of the *ced-3* genomic and cDNA sequences revealed that the *ced-3* gene has seven introns that range in size from 54 bp to 1195 bp (Figure 5A). The four largest introns, as well as sequences 5' of the

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start codon, were found to contain repetitive elements. Five types of repetitive elements were found, some of which have been previously characterized in non-coding regions of other *C. elegans* genes such as *fem-1* (Spence
5 et al., *Cell* 60:981-990 (1990)), *lin-12* (J. Yochem, personal communication), and *myoD* (Krause et al., *Cell* 63:907-919 (1990)) (Figure 4). Of these, repeat 1 was also found in *fem-1* and *myoD*, repeat 3 in *lin-12* and *fem-1*, repeat 4 in *lin-12*, and repeats 2 and 5 were
10 novel repetitive elements.

A combination of primer extension and PCR amplification was used to determine the location and nature of the 5' end of the *ced-3* transcript. Two primers (Pex1 and Pex2) were used for the primer
15 extension reaction. The Pex1 reaction yielded two major bands, whereas the Pex2 reaction gave one band. The Pex2 band corresponded in size to the smaller band from the Pex1 reaction, and agreed in length with a possible transcript that is trans-spliced to a *C.*
20 *elegans* splice leader (Bektesh, *Genes & Dev.*, 2:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence (Figure 4). The nature of the larger Pex1 band is unclear.

To confirm the existence of this trans-spliced
25 message in wild-type worms, total *C. elegans* RNA was PCR amplified using the SL1-Log5 and SL2-Log5 primer pairs, followed by a reamplification using the SL1-Oligo10 and SL2-Oligo10 primer pairs. The SL1 reaction yielded a fragment of the predicted length. The
30 identity of this fragment was confirmed by sequencing. Thus, at least some, if not most, of the *ced-3* transcript is trans-spliced to SL1. Based on this result, the start codon of the *ced-3* message was assigned to the methionine encoded at position 2232 of
35 the genomic sequence (Figure 4).

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26E02T" BCT4BBD
The DNA sequences of 12 EMS-induced *ced-3* alleles were also determined (Figure 4 and Table 3). Nine of the 12 are missense mutations. Two of the 12 are nonsense mutations, which might prematurely terminate the translation of *ced-3*. These nonsense *ced-3* mutants confirmed that the *ced-3* gene is not essential for viability. One of the 12 mutations is an alteration of a conserved splicing acceptor G, and another has a change of a 70% conserved C at the splice site, which could also generate a stop codon even if the splicing is correct. Interestingly, these EMS-induced mutations are in either the N-terminal quarter or C-terminal half of the protein. In fact, 9 of the 12 mutations occur within the region of *ced-3* that encodes the last 100 amino acids of the protein. Mutations are notably absent from the middle part of the *ced-3* gene (Figure 5).

Ced-3 Protein Contains A Region Rich in Serines

20 The Ced-3 protein is very hydrophilic and no significantly hydrophobic region can be found that might be a trans-membrane domain (Figure 6). The Ced-3 protein is rich in serine. From amino acid 78 to amino acid 205 of the Ced-3 protein, 34 out of 127 amino acids are serine. Serine is often the target of serine/ threonine protein kinases (Edelman, *Ann. Rev. Biochem.* 56:567-613 (1987)). For example, protein kinase C can phosphorylate serines when they are flanked on their amino and carboxyl sides by basic residues (Edelman, 1987 *supra*). Four of the serines in the Ced-3 protein are flanked by arginines (Figure 4). The same serine residues might also be the target of related Ser/Thr kinases.

35 To identify the functionally important regions of the Ced-3 protein, genomic DNAs containing the *ced-3*

genes from two related nematode species, *C. briggsae* and *C. vulgaris* were cloned and sequenced (Figure 7; Seq. ID #20 and 21). Sequence comparison of the three *ced-3* genes showed that the non-serine-rich region of the proteins is highly conserved. In *C. briggsae* and *C. vulgaris*, many amino acids in the serine-rich region are dissimilar compared to the *C. elegans* *Ced-3* protein (Figure 7). It seems that what is important in the serine-rich region is the overall serine-rich feature rather than the exact amino acid sequence.

This hypothesis is also supported by analysis of *ced-3* mutations in *C. elegans*: none of the 12 EMS-induced mutations is in the serine-rich region, suggesting that mutations in this region might not affect the function of the Ced-3 protein and thus, could not be isolated in the screen for *ced-3* mutants.

Genotype	DNA Injected	Avg. No. Cell Corpses (L1 Head)	No. Animals Scored
<i>ced-1; ced-4;</i> <i>unc-31; nEx1</i>	C10D8; C14G10	9.4	10
<i>ced-1; ced-4;</i> <i>unc-31; nEx7</i>	C10D8-5 C14G10	11.5	10
<i>ced-1; ced-4</i> <i>unc-31; nEx8</i>	C10D8-5 C14G10	11.5	10
<i>ced-1</i>	None	23	20
<i>ced-1; ced-4</i>	None	0.6	20

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Table 2
Sites of Mutations in the *ced-4* Gene

<u>Allele</u>	<u>Mutation</u>	<u>Nucleotide</u>	<u>Codon</u>	<u>Consequence</u>
n1162	C to T	1131	40	Q to ochre (TAA)
n2274	C to T	1428	139	R to opal (TGA)
n1920 & n2247	G to A	1744	first base of 5' splice donor of intron 3	Altered splicing
n2273	G to A	1929	first base of 3' splice acceptor of intron 3	Altered splicing
n1948	T to A	2117	258	I to N
n1947	C to T	2128	262	Q to amber
n1894	G to A	3131	401	W to opal (TGA)

Nucleotide and codon positions correspond to the numbering in Figure 1.

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Table 3
Sites of Mutations in the ced-3 Gene

<u>Allele</u>	<u>Mutation</u>	<u>Nucleotide</u>	<u>Codon</u>	<u>Consequence</u>
n1040	C to T	2310	27	L to F
n718	G to A	2487	65	G to R
n2433	G to A	5757	360	G to S
n1164	C to T	5940	403	Q to termination
n717	G to A	6297	-	Splice acceptor loss
n1949	C to T	6322	412	Q to termination
n1286	G to A	6342	428	W to termination
n1129	C to T	6434	449	A to V
n1165	C to T	6434	449	A to V
n2430	C to T	6485	466	A to V
n2426	G to A	6535	483	E to K
n1163	C to T	7020	486	S to F

Nucleotide and codon positions correspond to the numbering in Figure 4.

Table 4
Summary of Transformation Experiments
Using Cosmids in the ced-3 Region

<u>Cosmid</u> <u>injected</u>	<u>No. of non-Unc</u> <u>transformants</u>	<u>Ced-3</u> <u>phenotype</u>	<u>Strain name</u>
C43C9; C14G10	1	-	MT4302
W07H6; C14G10	3	-	MT4299
		-	MT4300
		-	MT4301
C48D1; C14G10	2	+	MT4298
		+	MT4303

Animals injected were of genotype: *ced-1(e1735); unc-31(e929)*
ced-3(n717).

26E06T" B2T42630

The expression of ced-3(+) transformants

Genotype	DNA injected	Average No. cell deaths in L1 head	No. Animals scored
<i>ced-1</i>	-	23	20
<i>ced-1; ced-3</i>	-	0.3	10
<i>ced-1; nIS1</i> <i>unc-31 ced-3</i>	C48D1; C14G10	16.4	20
<i>ced-1; unc-31</i> <i>ced-3; nIS1/+</i>		14.5	20
<i>ced-1; unc-31</i> <i>ced-3; nEX2</i>	C48D1; C14G10	13.2	10/14
		0	4/14
<i>ced-1; unc-31</i> <i>ced-3; nEX10</i>	C48D1-28; C14G10	12	9/10
		0	1 of 10
<i>ced-1; unc-31</i> <i>ced-3; nEX9</i>	C48D1-28; C14G10	12	10
<i>ced-1; unc-31</i> <i>ced-3; nEX11</i>	C48D1-43 C14G10	16.7	10/13
		Abnormal cell deaths	3/13
<i>ced-1; unc-31</i> <i>ced-3; nEX13</i>	pJ40; C14G10	13.75	4/4

Table 5 continued

<i>ced-1; unc-31</i> <i>ced-3; nEX17</i>	pJ107del128, pJ107del134 C14G10	23	12/14
		0	2/14
<i>ced-1; unc-31</i> <i>ced-3; nEX18</i>	pJ107del128, pJ107del1134 C14G10	12.8	9/10
		0	1/10
<i>ced-1; unc-31</i> <i>ced-3; nEX19</i>	pJ107del128, pJ107del134 G14G10	10.6	5/6
		0	1/6
<i>ced-1; unc-31</i> <i>ced-3; nEX16</i>	pJ107del112, pJ107del127 C14G10	7.8	12/12

Alleles of the genes used are *ced-1*(e1735), *unc-31*(e928), and *ced-3*(n717).

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. For example, functional equivalents of DNAs and RNAs may be nucleic acid sequences which, through the degeneracy of the genetic code, encode the same proteins as those specifically claimed.

Functional equivalents of proteins may be substituted or modified amino acid sequences, wherein the substitution or modification does not change the activity or function of the protein. A "silent" amino acid substitution, such that a chemically similar amino acid (e.g., an acidic amino acid with another acidic amino acid) is substituted, is an example of how a functional equivalent of a protein can be produced. Functional equivalents of nucleic acids or proteins can also be produced by deletion of nonessential sequences.

CLAIMS

1. Isolated DNA which is the *ced-3* gene.
2. Isolated DNA having the nucleotide sequence of Figure 4 (Seq. ID #18).
- 5 3. Isolated DNA encoding the amino acid sequence of Figure 4 (Seq. ID #19).
4. Isolated RNA encoded by the DNA of Claim 1.
5. Isolated protein encoded by the DNA of Claim 1.
- 10 6. Isolated protein having the amino acid sequence of Figure 4 (Seq. ID #19).
7. An antibody directed against the protein of Claim 6.
- 15 8. Isolated DNA which is a mutated *ced-3* or *ced-4* gene having a mutation which affects the activity of the gene.
9. The DNA of Claim 8, wherein the mutated *ced-4* gene is selected from the group consisting of:
 - a) *n1162*;
 - b) *n2274*;
 - 20 c) *n1920*;
 - d) *n2247*;
 - e) *n2273*;
 - f) *n1948*;
 - g) *n1947*; and
 - 25 h) *n1894*.

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10. The DNA of Claim 8, wherein the mutation in *ced-4* results in an alteration selected from the group consisting of:
- a) Q to termination at codon 40;
 - b) R to termination at codon 139;
 - c) I to N at codon 258;
 - d) Q to termination at codon 262;
 - e) W to termination at codon 401; and
 - f) an alteration in mRNA splicing resulting from a change at nucleotide 6297.
11. The DNA of Claim 8, wherein the mutation in *ced-4* is selected from the group consisting of:
- a) C to T at nucleotide 1131;
 - b) C to T at nucleotide 1428;
 - c) G to A at nucleotide 1929;
 - d) T to A at nucleotide 2117;
 - e) C to T at nucleotide 2128; and
 - f) G to A at nucleotide 3131.
12. The DNA of Claim 8, wherein the mutated *ced-3* gene is selected from the group consisting of:
- a) *n1040*;
 - b) *n718*;
 - c) *n2433*;
 - d) *n1164*;
 - e) *n717*;
 - f) *n1949*;
 - g) *n1286*;
 - h) *n1129*;
 - i) *n1165*;
 - j) *n2430*;
 - k) *n2426*; and
 - l) *n1163*.

13. The DNA of Claim 8, wherein the mutation in *ced-3* results in an alteration selected from the group consisting of:

- a) L to F at codon 27;
- 5 b) G to R at codon 65;
- c) G to S at codon 360;
- d) Q to termination at codon 403;
- e) Q to termination at codon 417;
- f) W to termination at codon 428;
- 10 g) A to V at codon 449;
- h) A to V at codon 466;
- i) E to K at codon 483;
- j) S to F at codon 486; and
- 15 k) an alteration in mRNA splicing at nucleotide 6297.

14. The DNA of Claim 8, wherein the mutation in *ced-3* is selected from the group consisting of:

- a) C to T at nucleotide 2310;
- b) G to A at nucleotide 2487;
- 20 c) G to A at nucleotide 5757;
- d) C to T at nucleotide 5940;
- e) G to A at nucleotide 6297;
- f) C to T at nucleotide 6322;
- 25 g) G to A at nucleotide 6342;
- h) C to T at nucleotide 6434;
- i) C to T at nucleotide 6485;
- j) G to A at nucleotide 6535; and
- k) C to T at nucleotide 7020.

15. Isolated RNA encoded by the DNA of Claim 8.

30 16. Isolated protein encoded by the DNA of Claim 8.

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thereby identifying a gene which is structurally related to a cell death gene selected from *ced-3* and *ced-4*.

- 5 24. The method of Claim 23, wherein the DNA is a gene library.
25. The method of Claim 23, wherein the nucleic acid probe further comprises degenerate oligonucleotides derived from the amino acid sequence of the product of the cell death gene.
- 10 26. A method for identifying a gene which is structurally related to a cell death gene selected from *ced-3* and *ced-4*, comprising the steps of:
- 15 1) combining nucleic acid with primers comprising portions of said cell death gene under conditions suitable for polymerase chain reaction; and
- 20 2) detecting specific DNA amplification, wherein specific DNA amplification produces a structurally related gene, or portion, thereby identifying a gene which is structurally related to a cell death gene selected from *ced-3* and *ced-4*.
- 25 27. The method of Claim 26, wherein the primers further comprise degenerate oligonucleotides derived from the amino acid sequence of the product of the cell death gene.

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28. A method for identifying a gene which is structurally related to a cell death gene selected from *ced-3* and *ced-4*, comprising the steps of:
- 5 a) combining an expression gene library with an antibody directed against the protein encoded by said cell death gene under conditions suitable for specific antibody-antigen binding of the antibody to antigens expressed from the gene library; and
 - 10 b) detecting specific antibody-antigen binding, wherein specific antibody-antigen binding indicates that a structurally related gene is present in the expression gene library,
 - 15 thereby identifying a gene which is structurally related to a cell death gene selected from *ced-3* and *ced-4*.
29. A bioassay for identifying a cell death gene, comprising the steps of:
- 20 a) using a gene and a nematode selected from a nematode having reduced activity of a cell death gene and a wild-type nematode to produce a transgenic nematode; and
 - 25 b) determining in said transgenic nematode an increase in cell deaths which occur during the development of the nontransgenic nematode, wherein an increase in cell deaths indicates the activity of a cell death gene, thereby identifying a cell death gene.
30. The bioassay of Claim 29, wherein the nematode underexpresses or expresses an inactivated form of a gene selected from *ced-3* and *ced-4*.
- 30

- b) constitutive activation of the cell death gene; and
- c) production of a mutated gene which does not cause cell death and which antagonizes the activity of functioning cell death genes.
- 5
37. A bioassay for identifying a gene which affects the activity of a cell death gene, comprising the steps of:
- 10 a) using a gene and a nematode containing a cell death gene to produce a transgenic nematode; and
- b) determining in said transgenic nematode a difference in cell deaths from cell deaths which occur during the development of the nontransgenic nematode, wherein a difference in cell deaths indicates a gene which affects the activity of a cell death gene,
- 15 thereby identifying a gene which affects the activity of a cell death gene.
- 20 38. The bioassay of Claim 37, wherein the cell death gene is selected from the group consisting of:
- a) a wild-type gene;
- b) an underexpressed gene;
- c) a gene having reduced activity;
- 25 d) an overexpressed gene; and
- e) a gene having hyperactivity.
39. The bioassay of Claim 37, wherein the gene is a component of an expression gene library.
40. An isolated gene identified by the bioassay of Claim 37.
- 30

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thereby identifying an agent which affects the activity of a cell death gene.

- 5 46. The bioassay of Claim 45, wherein the nematode expresses an endogenous cell death gene or a cell death gene which is a transgene.

47. The bioassay of Claim 46, wherein the cell death gene is *ced-3* or *ced-4*.

- 10 48. The bioassay of Claim 45, wherein the nematode overexpresses or underexpresses the cell death gene.

49. The bioassay of Claim 45, wherein the nematode expresses an inactivated or constitutively activated form of the cell death gene.

- 15 50. The bioassay of Claim 45, wherein the nematode underexpresses or expresses an inactivated form of a gene selected from *ced-3* and *ced-4*.

51. An agent identified by the bioassay of Claim 45.

- 20 52. The agent of Claim 47 which is selected from the group consisting of:
- 25 a) single stranded nucleic acid having all or a portion of the antisense sequence of the cell death gene which is complementary to the mRNA encoded by the gene;
- b) DNA encoding a); and
- c) an antagonist of the cell death gene.

53. A method for altering the occurrence of cell death, comprising altering in the cell the activity of a cell death gene.
54. The method of Claim 53, wherein the cell death gene is *ced-3* or *ced-4*.
55. The method of Claim 53, comprising exposing the cell to an agent which alters or mimics the activity of a cell death gene in the cell under conditions appropriate for activity of the agent.
56. The method of Claim 55, wherein the activity of the cell death gene is increased, comprising exposing the cell to an agent selected from the group consisting of:
- a) DNA comprising the cell death gene, or active portion thereof;
 - b) RNA encoded by the cell death gene, or active portion thereof;
 - c) protein encoded by the cell death gene, or active portion thereof;
 - d) an agent which is structurally similar to and mimics the activity of the protein encoded by the cell death gene;
 - e) DNA comprising a constitutively activated form of a cell death gene, or active portion thereof;
 - f) RNA encoded by the DNA of e), or active portion thereof;
 - g) protein encoded by the DNA of e), or active portion thereof;
 - h) an agent which is structurally similar to and mimics the activity of the protein encoded by the DNA of a); and

i) an agonist of the cell death gene,
under conditions appropriate for the activity of
the agent.

57. The method of Claim 55, wherein the activity of
the cell death gene is decreased, comprising
exposing the cell to an agent selected from the
group consisting of:
- a) single stranded nucleic acid having all or a
portion of the antisense sequence of the cell
death gene which is complementary to the mRNA
of the gene;
 - b) DNA which directs the expression of a);
 - c) a mutated cell death gene which does not
cause cell death and which antagonizes the
activity of the cell death gene;
 - d) RNA encoded by c);
 - e) protein encoded by c); and
 - f) an antagonist of the cell death gene,
under conditions appropriate for the activity of
the agent.
58. A method for reducing the proliferative capacity
or size of a population of cells, comprising
increasing the activity of a cell death gene in
the cells.
59. The method of Claim 58, wherein the cells are
selected from:
- a) cancerous cells;
 - b) infected cells;
 - c) cells producing autoreactive
antibodies; and
 - d) hair follicle cells.

60. The method of Claim 58, wherein the cell death gene is selected from the group consisting of:
- a) *ced-3*;
 - b) a cell death gene which is structurally related to *ced-3*; and
 - c) a gene which is functionally related to *ced-3*.
61. The method of Claim 58, wherein the cell death gene is selected from the group consisting of:
- a) *ced-4*;
 - b) a cell death gene which is structurally related to *ced-4*; and
 - c) a gene which is functionally related to *ced-4*.
62. A method for treating a condition characterized by cell deaths, comprising decreasing the activity of a cell death gene.
63. The method of Claim 62, wherein the condition is selected from the group consisting of:
- a) myocardial infarction;
 - b) stroke;
 - c) degenerative disease;
 - d) traumatic brain injury;
 - e) hypoxia;
 - f) pathogenic infection;
 - g) aging; and
 - h) hair loss.

- 5 64. A method for treating a parasitic infection of a host animal, comprising administering an agent which increases the activity of a cell death gene specific to the parasite and which does not harm the host animal.
65. The method of Claim 64, wherein the parasite is a nematode.
- 10 66. A method for incapacitating or killing a pest, comprising increasing the activity of a cell death gene in the pest.
- 15 67. A method of biological containment of a recombinant organism, comprising introducing in the organism nucleic acid which is able to direct the expression of an agent which increases the activity of a cell death gene in the organism under predetermined conditions, thereby incapacitating or killing the recombinant organism.
- 20 68. The method of Claim 67, wherein the agent kills the recombinant organism upon completion of a desired task by the organism.

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Described herein are genes shown to be essential for programmed cell death in *C. elegans*, their encoded products (RNA and polypeptides), antibodies directed against the encoded polypeptides; probes for identifying structurally related genes and bioassays for identifying functionally related cell death genes from various organisms; methods and agents for altering (increasing or decreasing) the activity of the cell death genes and, thus, of altering cell death; and uses therefor. Specifically, two genes shown to be essential for almost all of the cell deaths which occur in the development of *C. elegans*, referred to as *ced-3* and *ced-4*, have been cloned, sequenced and characterized.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CLONING, SEQUENCING AND CHARACTERIZATION OF TWO CELL DEATH GENES
AND USES THEREFOR

the specification of which (check one)

☐ is attached hereto.

☒ was filed on November 20, 1992 as
Application Serial No. 07/979,638
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

			Priority Claimed	
(Number)	(Country)	(Day/Month/Year filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>

2025-11-13 10:00:00

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

07/897,788	June 12, 1992	pending
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)
<hr/>		
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states.

David E. Brook	Registration No. 22,592
James M. Smith	Registration No. 28,043
Leo R. Reynolds	Registration No. 20,884
Richard A. Wise	Registration No. 18,041
Patricia Granahan	Registration No. 32,227
Mary Lou Wakimura	Registration No. 31,804
Thomas O. Hoover	Registration No. 32,470
Alice O. Carroll	Registration No. 33,542
Pei-Pei C. Smith	Registration No. 36,280

all of Hamilton, Brook, Smith and Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02173;

and

Send correspondence to:	Patricia Granahan, Esq.
	HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
	Two Militia Drive
	Lexington, Massachusetts 02173-4799
Direct telephone calls to:	Patricia Granahan, Esq.
	(617) 861-6240

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole
or first inventor H. Robert Horvitz
Inventor's
Signature *H. Robert Horvitz* Date JANUARY 26, 1993
Residence 80 Wendell Street, #2
Cambridge, Massachusetts 02138
Citizenship U.S.A.
Post Office Address same

Full name of second joint
inventor, if any Junying Yuan
Second Inventor's
Signature *Junying Yuan* Date 1/26/93
Residence 22 Fairlee Road
Newton, Massachusetts 02168
Citizenship China
Post Office Address same

Full name of third joint
inventor, if any Shai Shaham
Third Inventor's
Signature *Shai Shaham* Date 1/26/93
Residence 132 Commonwealth Avenue, #9
Boston, Massachusetts 02116
Citizenship Israel and U.S.A.
Post Office Address same

Full name of fourth joint
inventor, if any _____
Fourth Inventor's
Signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

2025-01-26 14:30:00

T n2274

1381 ATGTTCCAAAACAAATGACATGCTATATTCGAGAGTATCACGTGGATCGAGTGATCAAAA 1440
 V P K Q M T C Y I R E Y E V D R V I K K
 130 140

Intron 1

1441 AGCTCGACGAGATGTGTGATTAGGTGAGAAAAGCTCTCGTGTATTATTATAATC
 L D E M C D L D
 150

1501 TTGCTTAAACTTCAGACTCTTTTCTGTTTCTACACGGCCGAGCTGGATCCGAAAAT 1560
 S F F L F L H G R A G S G K S
 160

Intron 2

1561 CAGTAATTGCATCACAAGCTCTTTCGAAATCTGACCAACTTATTGGAATGTGAGTGGTAT 1620
 V I A S Q A L S K S D Q L I G I
 170 180

1621 TATCTGAATCTACGGATCTTCTATTACAGAAATTATGATTCAATCGTTGGCTCAA 1680
 N Y D S I V W L K
 190

1681 AGATAGTGAACAGCTCCAAATCTACATTGATTATTATTACGGATATTTTGCTGATGCT 1740
 D S G T A P K S T F D L F T D I L L M L
 200 210

A n1920/n2247
 Intron 3

1741 AAAGTGAGTGAATAGAGTGCATGTAACATTCAGCATGATTTTGAATATGAAAATTGA 1800
 K
 CCTGGTTAGCTTTTAATTTGATATTTCTGACGCTTGCATGTTTTGTGTGTTGAAGACG
 1801 AGCCCGTGTGTGAGCGACACGGATGACTCGCATTGATCACCAGCTTCATTAAACCGTGT 1860
 1861

A n2273

1921 TCTTTCAAGCAGCGAAGACGATCTTCTCAATTTCCCATCGGTGGAGCATGTCACGTCAGT 1980
 S E D D L L N F P S V E H V T S V
 220

Intron 4

1981 TGTAATCAAAAGGATGTAAGTTGCTTGCCGATTCTGGTACAATATCTTAAATTATTGGT 2040
 V L K R M
 230

2041 TTTTAGATCTGCAACGCACTCATTGATCGTCCAAATACCTTTATTCGTATTGATGACGTA 2100
 I C N A L I D R P N T L F V F D D V
 240 250

A n1948 T n1947

2101 GTTCAAGAAGAAACAATTCGTTGGGCTCAGGAGCTACGCTTCGATGTCTTGTAAGTACT 2160
 V Q E E T I R W A Q E L R L R C L V T T
 260 270

2161 CGTGACGTGGAAATATCAAAATGCTGCTTCTCAAACATGCGAATTCATTGAAGTGACATCA 2220
 R D V E I S N A A S Q T C E F I E V T S
 280 290

FIGURE 1

TTGAAAATCGATGAATGTTATGATTTTCTAGAAGCTTATAGGAATGCCGATGCCGTGTTGGA
2281 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2280

L E I D E C Y D F L E A Y G M P M P V G
300 310

Tc4 n1416
↓

GA AAAAGAAGAAGATGTGCTTAATAAAACAATCGAACTAAGCAGTGGAAATCCAGCAACG
2281 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2340

E K E E D V L N K T I E L S S G N P A T
320 330

Intron 5
CTTATGATGTTTTTCAAGTCTTGTGAACCGAAAAACATTTGAAAAGTGAGTGGGACATACC
2341 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2400

L M M F F K S C E P K T F E K
330

AATTGAGACTTTTTAAAAATAATTTATTCTACAATAAAAGTTAATCAAAAAGTTTTCATAGC
2401 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2460

TGATTGTCTTTAAATTTTACGAATTGAGGATCAAAATCAAGAATTAGGATCCTGGCAGCA
2461 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2520

GAGAAAACCTGTGTAGCTACCGTACCCGAGAGATTTTCTTGATATTGCCATCGATTTAAT
2521 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2580

TTTTTAAGAAAATTATCGTTTTACATAATTGAACAAGAGATACACGGTCTCGACCCGACG
2581 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2640

GAAATTTTTTAAATGAAAGCGAGTATGAGCCTGTTTTCATTATTTTCGATTTTCTCTTG
2641 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2700

TTGTTTCTTTTATTAAAGCCTTTTATTTTGAACAAGTCTAAAAATATTAAAACTGA
2701 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2760

ATAAAATATTTAAAAAAAATCAAGTAAAAATAGAAAAACAGCAAGGCTGGAGACTACTGTA
2761 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2820

CTTCTTAAATCCGCATACTCTTTTATTTAATCATTTTCCGGAATGTCGAAACGAAATAA
2821 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2880

TACATTTTGTAGTCCAAAATCGCTAGGTATATTCTTAAATTTATCAAAACATTTTGATTCA
2881 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2940

GAATGGCACAGCTTAATAACAAATTGAAAGTCGAGGATTAGTCGGTGTGTAATGTATCA
2941 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3000

M A Q L N N K L E S R G L V G V E C I T
340 350

CCCCTTACTCGTACAAAGTCACTCGCAATGGCTCTTCAAAGATGTGTTGAAGTTTTGTCAG
3001 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3060

P Y S Y K S L A M A L Q R C V E V L S D
360 370

ATGAGGATCGAAGTGCTCTTGCTTTTCGAGTTGTGATGCCTCCTGGAGTTGATATACCCG
3061 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3120

E D R S A L A F A V V M P P G V D I P V
380 390

A n1894
↑

TCAAGCTATGGTTCATGTGTTATTCCAGTTGATATTGTTCAAATGAAGAAGAACAATTGG
3121 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3180

K L W S C V I P V D I C S N E E E Q L D
400 410

Intron 6
ATGATGAAGTTGCGGATCGGTTGAAAAGACTCAGCAAGTATGAGTCTTGAAATTTGAAGA
3181 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3240

D E V A D R L K R L S K
420

TTTAAATTAACACTTAAAAATTCAGACGTGGAGCTCTTCTCAGTGGAAAACGAATGCCCG
3241 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 3300

R G A L L S G K R M P V
430 440

FIGURE 1

3301 TTTTGACATTCAAAATTGATCATATTATCCATAIGTTCTTGAAACACGTCGTTGATGCAC 3360
 L T F K I D H I I H M F L K H V V D A Q
 450 460
 Intron 7
 3361 AACTATCGCCGTATGCTGAAAATGTCTCAACTTTCAATTAAATTTTAAATTTTCAGAAAT 3420
 T I A N
 3421 GGAATCTCAATTCTCGAGCAGCGTCTTCTTGAAATAGGAAACAATAATGTATCAGTACCG 3480
 G I S I L E Q R L L E I G N N N V S V P
 470 480
 3481 GAGCGACATATACCATCACATTTCCAAAAATTCGTCGTTTCATCAGCCAGTGAGATGTAT 3540
 E R H I P S H F Q K F R R S S A S E M Y
 500 510
 3541 CCAAAAACCTACAGAAGAACTGTGATCCGTCCTGAAGACTTCCCAAAGTTCATGCAATTG 3600
 P K T T E E T V I R P E D F P K F M Q L
 520 530
 3601 CACCAGAAATTTCTATGACTCCCTCAAAAATTTTGCATGCTGTTAAACCTATCGTGACA 3660
 H Q K F Y D S L K N F A C C *
 540
 3661 ATATTGCCGTGATATTTCCCTCGAAATACGTTTATACTTTTTTCGCACGAGTTTCTCATT 3720
 TTTTCATTGTACTTGTTTTATTCTCTCCAAAATTCAGATCTATCCCAAATGTTCTTA
 3721 AATTTAATGTTTTCTACAGATACTCAACACATCTGTTTCACTCATCCTTGCTTTTTT 3780
 3781 TTTCAAATATATTCAGTTTCTTTATAATTTTAATTAATCGAATTAATACATTCACGTAA 3840
 3841 AGAATTTCTGGACTATTATTTATCGCATCCAAATGATTTATTCCTATTGTTTCGAAAC 3900
 3901 TTCCAAATTGATCATTTTTTAACACGCGCTCATTAAATGAAAGTCGTACTTTTAGTCTCG 3960
 3961 AACATGAAGTAAGTTATTTCTGTGTTCTAAATTCAAAGTGCATTCCAAAAGGACATTG 4020
 4021 ATGAGTTTTACGAAAACCGTAATTTTACAATTTCCTTTCAGTTTGAAGATGTTTCGAT 4080
 4081 TTCTTTCTCTGTTGGCGTCATTACTACATTGCTTTGCTGCTTCACTTTATCGAGATTC 4140
 4141 TTGCCATCAATGGAGTTCATCTAGACCGATAGCAGTCTTCATATCATTATCCCTGTATA 4200
 4201 TTGTACTGTTTCAGTATTTTAACTTATCGATTACGTACTATATTCAGTGGTTCAGTGT 4260
 4261 TCGGTCAATGGGTGACACGTGCTCGACGANNAAATTTCAACGAACGAATCTCCTAGTCA 4320
 4321 CTTATCAACCAAGAGCCCTCACCCATG 4380
 4381 4407

FIGURE 1

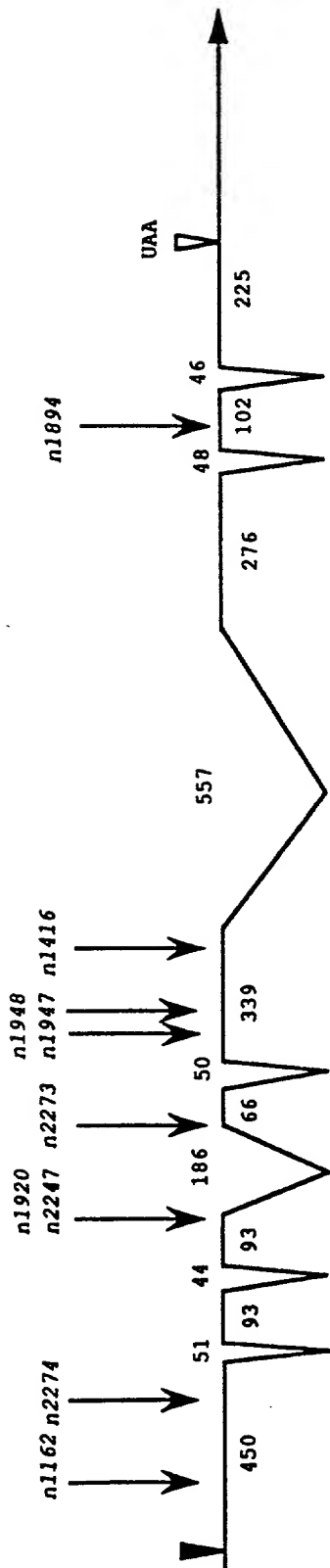


FIGURE 2

	10	12	14	18	21
	X	Y	Z	-X	-Z
Calcium-binding loop consensus	D	N	S	T	E
		D	N	S	
			D	E	
				Q	
				D	
				N	
EF-hand consensus	<u>O * O * O G * * O * * E</u>				
ced-4 sequence 1	Y	N	N	Q	S
sequence 2	S	L	E	I	D
Parvalbumin (carp)	D	Q	D	K	S
(hake)	D	Q	D	K	D
(ray)	D	S	D	G	D
SCBP (<i>Amphioxus I</i>)	D	I	N	K	D
ICaBP (bovine)	A	K	E	G	D
	D	K	N	G	D
Troponin C (rabbit)	D	A	D	G	G
	D	E	D	G	S
	D	R	N	A	D
	D	K	N	N	D
Calmodulin (bovine)	D	K	D	G	N
Trypsinogen	L	G	E	D	N
Fibrinogen	D	N	D	N	D
Villin	G	V	D	P	S
GBP	D	L	N	K	D

FIGURE 3

ced-3 Genomic Sequence

```

1  AGATCTGAAATAAGGTGATAAATTAATAAATTAAGTGTATTTCTGAGGAAATTTGACTGT 60
   +-----+-----+-----+-----+-----+
61  TTTAGCACAAATTAATCTTGTTTCAGAAAAAAGTCCAGTTTCTAGATTTTTCGGTCTTA 120
   +-----+-----+-----+-----+-----+
121 TTGTCGAATTAATATCCCTATTATCACTTTTTCATGCTCATCCTCGAGCGGCACGTCTC 180
   +-----+-----+-----+-----+-----+
181 AAAGAATTGTGAGAGCAAACGCGCTCCCATTGACCTCCACACTCAGCCGCCAAAAACAAAC 240
   +-----+-----+-----+-----+-----+
241 GTTCGAACATTTCGTGTGTGTGCTCCTTTTCCGTTATCTTGCACTCATCTTTTGTCTGTTT 300
   +-----+-----+-----+-----+-----+
301 TTTTCTTTGTTCTTTTGTGTAACGTGTTGCTAAGCAATTATTACATCAATTGAAGAAAA 360
   +-----+-----+-----+-----+-----+
361 GGCTCGCCGATTTATTGTTGCCAGAAAGATTCTGAGATTCTCGAAGTCGATTTTATAATA 420
   +-----+-----+-----+-----+-----+
421 TTTAACCTTGGTTTTTGCATTGTTTCGTTTAAAAAAACCAGTTTATGTGAAAAACGAT 480
   +-----+-----+-----+-----+-----+
481 TAGTTTACTAATAAACTACTTTTAAACCTTTACCTTTACCTCACCGCTCCGTGTTTCATG 540
   +-----+-----+-----+-----+-----+
541 GCTCATAGATTTTCGATACTCAAATCCAAATAAATTTACGAGGGCAATTAATGTGAAA 600
   +-----+-----+-----+-----+-----+
601 CAAAAACAATCCTAAGATTTCCACATGTTTGACCTCTCCGGCACCTTCTTCCTTAGCCCC 660
   +-----+-----+-----+-----+-----+
661 ACCACTCCATCACCTCTTTGGCGGTGTTCTTCGAAACCCACTTAGGAAAGCAGTGTGTAT 720
   +-----+-----+-----+-----+-----+
721 CTCATTTGGTATGCTCTTTTCGATTTTATAGCTCTTGTGCAATTTCAATGCTTTAAAC 760
   +-----+-----+-----+-----+-----+
781 AATCCAAATCGCATTATATTTGTGCATGGAGGCAAATGACGGGGTTGGAATCTTAGATGA 840
   +-----+-----+-----+-----+-----+
841 GATCAGGAGCTTTCAGGGTAAACGCCCGGTTTCAATTTGTACCACATTTTCATCTTTTCT 900
   +-----+-----+-----+-----+-----+
901 GTCGTCCTTGGTATCCTCAACTTGTCGGGTTTTGTTTTCGGTACACTCTTCCGTGATGC 960
   +-----+-----+-----+-----+-----+
961 CACCTGTCTCCGTCTCAATTATCGTTTAGAAATGTGAAGTGTCCAGATGGGTGACTCATA 1020
   +-----+-----+-----+-----+-----+
1021 TTGCTGCTGCTACAATCCACTTTCTTTTCTCATCGGCAGTCTTACGAGCCCATCATAAC 1080
   +-----+-----+-----+-----+-----+
1081 TTTTTTTTCGCGAAATTTGCAATAAACCGGCCAAAACTTTCTCAAATTGTTACGCAA 1140
   +-----+-----+-----+-----+-----+
1141 TATATACAATCCATAAGAATATCTTCTCAATGTTTATGATTTCTTCGACGACTTTCTCT 1200
   +-----+-----+-----+-----+-----+
1201 TCGTGTGCTAACATCTTATTTTATAATATTTCCGCTAAAATTCGATTTTGTAGTATTA 1260
   +-----+-----+-----+-----+-----+
1261 ATTTATCGTAAAATTATCATAATAGCACCGAAACTACTAAAAATGGTAAAAGCTCCTTT 1320
   +-----+-----+-----+-----+-----+

                                     Repeat 1
                                     -----
1321 TAAATCGGCTCGACATTATCGTATTAAGGAATCACAAAATTCTGAGAATGCGTACTGCGC 1380
   +-----+-----+-----+-----+-----+

-----
1381 AACATATTTGACGGCAAAATATCTCGTAGCGAAACTACAGTAATTCTTTAAATGACTAC 1440
   +-----+-----+-----+-----+-----+

                                     Repeat 1
                                     ----->
1441 TGTAGCGCTTGTGTCGATTTACGGGCTCAATTTTGAAAATAATTTTTTTTTCGAATTT 1500
   +-----+-----+-----+-----+-----+

```

FIGURE 4

2581 TTTTAAAGTTCGGCGCAAAAGCAAGGGTCTCACGGAAAAAGAGGCGGATCGTAATTTT 2640
 -----+-----+-----+-----+-----+-----+-----+
 2641 GCAACCCACCGGCACGGTTTTTTCCTCCGAAATCGGAAATTATGCACCTTCCCAAATAT 2700
 -----+-----+-----+-----+-----+-----+-----+
 2701 TTGAAGTGAAATATATTTTATTTACTGAAAGCTCGAGTGATTATTTATTTTAACTA 2760
 -----+-----+-----+-----+-----+-----+-----+
 2761 ATTTTCGTGGCGCAAAAGGCCATTTTGTAGATTGCGGAAATACTTGTACACACACAC 2820
 -----+-----+-----+-----+-----+-----+-----+

 2821 ACACACATCTCCTTCAAATATCCCTTTTCCAGTGTGACTCGAATGCTGTGAATTCGA 2880
 -----+-----+-----+-----+-----+-----+-----+
 V D S N A V E F E
 100

 2881 GTGTCCAATGTCACCGGCAAGCCATCGTCGGAGCCGCGCATTGAGCCCCGCCGGCTACAC 2940
 -----+-----+-----+-----+-----+-----+-----+
 C P M S P A S H R R S R A L S P A G Y T
 110 120

 2941 TTCACCGACCCGAGTTCACCGTGACAGCGTCTCTTCAGTGTCATCATTCACTTCTTATCA 3000
 -----+-----+-----+-----+-----+-----+-----+
 S P T R V H R D S V S S V S S F T S Y Q
 130 140

 3001 GGATATCTACTCAAGAGCAAGATCTCGTTCTCGATCGCGTGCACTTCATTCATCGGATCG 3060
 -----+-----+-----+-----+-----+-----+-----+
 D I Y S R A R S R S R S R A L H S S D R
 150 160

 3061 ACACAATTATTCATCTCCTCCAGTCAACGCATTTCAGCCAACTTGATGTTGATGCG 3120
 -----+-----+-----+-----+-----+-----+-----+
 H N Y S S P P V N A F P S Q P S
 170

 Repeat 1
 -----+-----+-----+-----+-----+-----+-----+
 3121 AACACTAAATTCTGAGAATGCGCATTACTCAACATATTTGACGCGCAAATATCTCGTAGC 3180
 -----+-----+-----+-----+-----+-----+-----+

 3181 GAAAAATACAGTAACCCCTTAAATGACTATTGTAGTGTGATTTACGGGCTCGATTTTCG 3240
 -----+-----+-----+-----+-----+-----+-----+

 ==>
 3241 AAACGAATATATGCTCGAATTGTGACAACGAATTTTAAATTGTGATTTTGTGTTTCTT 3300
 -----+-----+-----+-----+-----+-----+-----+

 Repeat 1
 -----+-----+-----+-----+-----+-----+-----+
 3301 TTGATATTTTGTATCAATTAATAAATTATTTCCGTAAACAGACACCAGCGCTACAGTACT 3360
 -----+-----+-----+-----+-----+-----+-----+

 -----+-----+-----+-----+-----+-----+-----+
 3361 CTTTTAAAGAGTTACAGTAGTTTTCGCTTCAAGATATTTTGAAAAGAATTTTAAACATTT 3420
 -----+-----+-----+-----+-----+-----+-----+

 3421 TGAAAAAAATCATCTAACATGTGCCAAAACGCTTTTTTCAAGTTTCGCAGATTTTTTGA 3480
 -----+-----+-----+-----+-----+-----+-----+

FIGURE 4

[illegible]

S A N S S F
180

-4-

```

4321 TCACCGGATGCTCTTCTCTCGGATACAGTTCAAGTCGTAATCGCTCATTGAGCAAAGCTT 4380
-----+-----+-----+-----+-----+-----+-----+-----+
      T G C S S L G Y S S S R N R S F S K A S
              190                               200

4381 CTGGACCAACTCAATACATATTCCATGAAGAGGATATGAACCTTGTGCGATGCACCAACCA 4440
-----+-----+-----+-----+-----+-----+-----+-----+
      G P T Q Y I F H E E D M N F V D A P T I
              210                               220

4441 TAAGCCGTGTTTTTCGACGAGAAAAACCATGTACAGAACTTCTCGAGTCCTCGTGAATGT 4500
-----+-----+-----+-----+-----+-----+-----+-----+
      S R V F D E K T M Y R N F S S P R G M C
              230                               240

4501 GCCTCATCATAATAATGAACACTTTGAGCAGATGCCAACACGGAATGGTACCAAGGCCG 4560
-----+-----+-----+-----+-----+-----+-----+-----+
      L I I N N E H F E Q M P T R N G T K A D
              250                               260

4561 ACAAGGACAATCTTACCAATTTGTTTCAGATGCATGGGCTATACGGTTATTTGCAAGGACA 4620
-----+-----+-----+-----+-----+-----+-----+-----+
      K D N L T N L F R C M G Y T V I C K D N
              270                               280

      | intron 4
4621 ATCTGACGGGAAGGTTACGGCGAAATTATATTACCCAAACGCGAAATTTGCCATTTTGCG 4680
-----+-----+-----+-----+-----+-----+-----+-----+
      L T G R

      Repeat 3
      =====>
4681 CCGAAAATGTGGCGCCCGGTCTCGACACGACAATTTGTGTTAAATGCAAAAATGTATAAT 4740
-----+-----+-----+-----+-----+-----+-----+-----+
4741 TTTGCAAAAACAAAATTTTGAACCTCCGCGAAAATGATTACCTAGTTTCGAAAATTTTC 4800
-----+-----+-----+-----+-----+-----+-----+-----+
4801 GTTTTTTCCGGCTACATTATGTGTTTTTCTTAGTTTTTCTATAATATTGATGTAAAAA 4860
-----+-----+-----+-----+-----+-----+-----+-----+
4861 ACCGTTTGTAATTTTTCAGACAATTTTCCGCATACAAAACCTTGATAGCACGAAATCAATT 4920
-----+-----+-----+-----+-----+-----+-----+-----+
4921 TTCTGAATTTTCAAATTATCCAAAATGCACAATTTAAAATTTGTGAAAATTGGCAAAC 4980
-----+-----+-----+-----+-----+-----+-----+-----+
4981 GGTGTTTCAATATGAAATGTATTTTTTAAAACTTTAAAAACCACTCCGAAAAGCAATAA 5040
-----+-----+-----+-----+-----+-----+-----+-----+
5041 AAATCAAAACAACGTCACAATTCAAATCAAAAGTTATTCATCCGATTGTGTTATTTTGT 5100
-----+-----+-----+-----+-----+-----+-----+-----+
5101 CAAAATTTGAAAAATCATGAAGGATTAGAAAAGTTTATAACATTTTCTAGATTTT 5160
-----+-----+-----+-----+-----+-----+-----+-----+
5161 TCAAAATTTTAAAAAATCGAGAAAAAGAGAATGAAAAATCGATTTTAAAAATATCC 5220
-----+-----+-----+-----+-----+-----+-----+-----+

      Repeat 3
      <=====
5221 ACAGCTTCGAGAGTTTGAAATTACAGTACTCCTTAAAGGCGCACACCCCATTTGCATTGG 5280
-----+-----+-----+-----+-----+-----+-----+-----+

      =====
5281 ACCAAAAATTTGTCGTGTCGAGACCAGGTACCGTAGTTTTTGTGCAAAAATTGCACCAT 5340
-----+-----+-----+-----+-----+-----+-----+-----+
5341 TGGACAATAAACCTTCCTAATCACCAAAAAGTAAAATTGAAATCTTCGAAAAGCCAAAAA 5400
-----+-----+-----+-----+-----+-----+-----+-----+

```

FIGURE 4

5401 ATTCAAAAAAAAAAGTCGAATTTTCGATTTTTTTTTTGGTTTTTTGGTCCCAAAACCAAAA 5460
 -----+-----+-----+-----+-----+-----+-----+-----+
 5461 AAATCAATTTTCTGCAAAATACCAAAAAGAAACCCGAAAAAATTCCCAGCCTTGTTCCT 5520
 -----+-----+-----+-----+-----+-----+-----+-----+
 5521 AATGTAAACTGATATTTAATTTCCAGGGAATGCTCCTGACAATTCGAGACTTTGCCAAAC 5580
 -----+-----+-----+-----+-----+-----+-----+-----+
 G M L L T I R D F A K H
 290 300
 5581 ACGAATCACACGGAGATTCTGCGATACTCGTGATTCTATCACACGGAGAAGAGAATGTGA 5640
 -----+-----+-----+-----+-----+-----+-----+-----+
 E S H G D S A I L V I L S H G E E N V I
 310 320
 5641 TTATTGGAGTTGATGATATACCGATTAGTACACAGAGATATATGATCTTCTCAACGCGG 5700
 -----+-----+-----+-----+-----+-----+-----+-----+
 I G V D D I P I S T H E I Y D L L N A A
 330 340
 A(n2433)
 5701 CAAATGCTCCCCGTCTGGCGAATAAGCCGAAATCGTTTTTGTGCAGGCTTGTGCGAGGCG 5760
 -----+-----+-----+-----+-----+-----+-----+-----+
 N A P R L A N K P K I V F V Q A C R G E
 350 360
 5761 GTTCGTTTTTTATTTTAATTTTAATATAAATATTTTAAATAAATTCATTTTCAGAACGTC 5820
 -----+-----+-----+-----+-----+-----+-----+-----+
 R R
 5821 GTGACAATGGATTCCCAGTCTTGGATTCTGTGCGAGGAGTTCCTGCATTTCTTCGTCGTG 5880
 -----+-----+-----+-----+-----+-----+-----+-----+
 D N G F P V L D S V D G V P A F L R R G
 370 380
 T(n1165)
 5881 GATGGGACAATCGAGACGGGCCATTGTTCAATTTTCTTGGATGTGTGCGGCCGCAAGTTC 5940
 -----+-----+-----+-----+-----+-----+-----+-----+
 W D N R D G P L F N F L G C V R P Q V Q
 390 400
 | intron 6
 5941 AGGTTGCAATTTAATTTCTTGAATGAGAATATTCCTTCAAAAAATCTAAATAGATTTT 6000
 -----+-----+-----+-----+-----+-----+-----+-----+
 6001 ATTCCAGAAAGTCCCGATCGAAAAATTGCGATATAATTACGAAATTGTGATAAATGAC 6060
 -----+-----+-----+-----+-----+-----+-----+-----+
 Repeat 4
 6061 AAACCAATCAGCATCGTCGATCTCCGCCCACTTCATCGGATTGGTTTGAAAGTGGGCGGA 6120
 -----+-----+-----+-----+-----+-----+-----+-----+
 ----->
 6121 GTGAATTGCTGATTGGTGCAGTTTTTCAGTTTAGAGGGAATTTAAAAATCGCCTTTTCGA 6180
 -----+-----+-----+-----+-----+-----+-----+-----+
 6181 AAATTAATAAATTGATTTTTTCAATTTTTTCGAAAAATATTCGATTATTTTATATTCTTT 6240
 -----+-----+-----+-----+-----+-----+-----+-----+

FIGURE 4

A(n717)
 |
 GGAGCGAAAGCCCCGTCTCTGTAAACATTTTTTAAATGATAATTAATAAATTTTTGCAGCAA
 6241 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6300
 Q
 T(n1949)
 |
 GTGTGGAGAAAGAAGCCGAGCCAAAGCTGACATTCTGATTTCGATACGCAACGACAGCTCAA
 6301 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6360
 V W R K K P S Q A D I L I R Y A T T A Q
 410 420
 A(n1286)
 |
 TATGTTTCGTGGAGAAACAGTGCTCGTGGATCATGGTTCATTCAAGCCGCTCTGTGAAGTG
 6361 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6420
 Y V S W R N S A R G S W F I Q A V C E V
 430 440
 T(n1129,n1164)
 |
 TTCTCGACACACGCAAAGGATATGGATGTTGTTGAGCTGCTGACTGAAGTCAATAAGAAG
 6421 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6480
 F S T H A K D M D V V E L L T E V N K K
 450 460
 T(n2430) A(n2426)
 | | intron 7
 GTCGCTTGTGGATTTCAGACATCACAGGGATCGAATATTTTGAAACAGATGCCAGAGGTA
 6481 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6540
 V A C G F Q T S Q G S N I L K Q M P E
 470 480
 Repeat 5
 CTTGAAACAAACAATGCATGTCTAACTTTTAAGGACACAGAAAAATAGGCAGAGGCTCCT
 6541 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6600
 =====>
 TTTGCAAGCCTGCCGCGCGTCAACCTAGAATTTTAGTTTTTAGCTAAAATGATTGATTTT
 6601 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6660
 GAATATTTTATGCTAATTTTTTTCGTTAAATTTTGAAATAGTCACTATTTATCGGGTTT
 6661 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6720
 CCAGTAAAAAATGTTTATTAGCCATTGGATTTTACTGAAAACGAAAATTTGTAGTTTTTC
 6721 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6780
 AACGAAATTTATCGATTTTAAATGTAAAAAAAATAGCGAAAATTACATCAACCATCAA
 6781 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6840
 GCATTTAAGCCAAAATTGTTAACTCATTTAAAAATTAATTCAAAGTTGTCCACGAGTATT
 6841 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 6900
 Repeat 5
 <=====

FIGURE 4

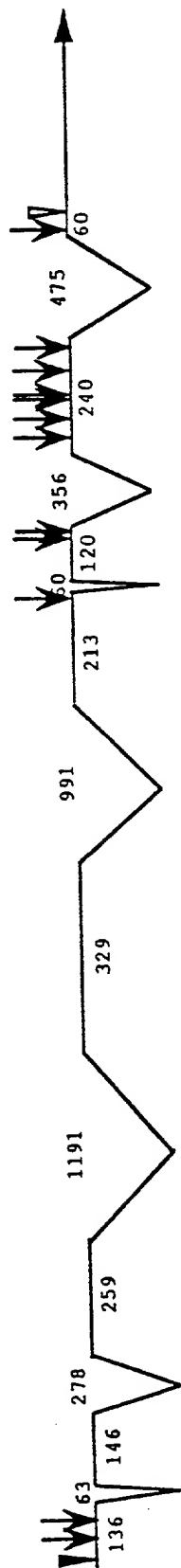


FIGURE 5A

ced-3 Mutations are Clustered

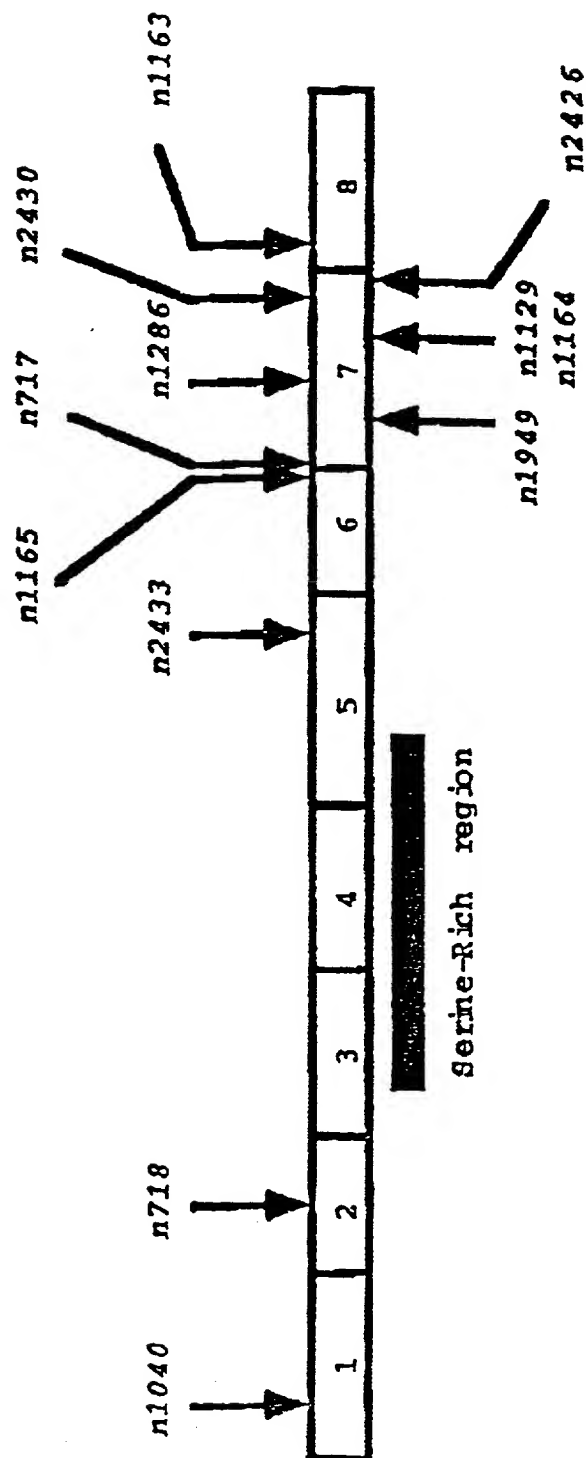


FIGURE 5B

FIGURE 6

Figure 1 consists of 12 bar charts, labeled (a) through (l), each representing a different fish species. The y-axis for all charts is 'Percentage of total catch' ranging from 0 to 100. The x-axis for all charts is 'Year' from 1990 to 2001. The species and their corresponding data are as follows:

- (a) Atlantic croaker: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (b) Striped bass: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (c) Blue crab: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (d) Atlantic menhaden: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (e) Weakfish: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (f) Spot: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (g) Bay anchovy: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (h) Atlantic silverside: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (i) Atlantic herring: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (j) Atlantic tomcod: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (k) Atlantic silverside: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.
- (l) Atlantic croaker: 1990: 100%, 1991: 100%, 1992: 100%, 1993: 100%, 1994: 100%, 1995: 100%, 1996: 100%, 1997: 100%, 1998: 100%, 1999: 100%, 2000: 100%, 2001: 100%.

Line 1 *C. elegans*
Line 2 *C. briggsae*
Line 3 *C. vulgaris*

FIGURE 7

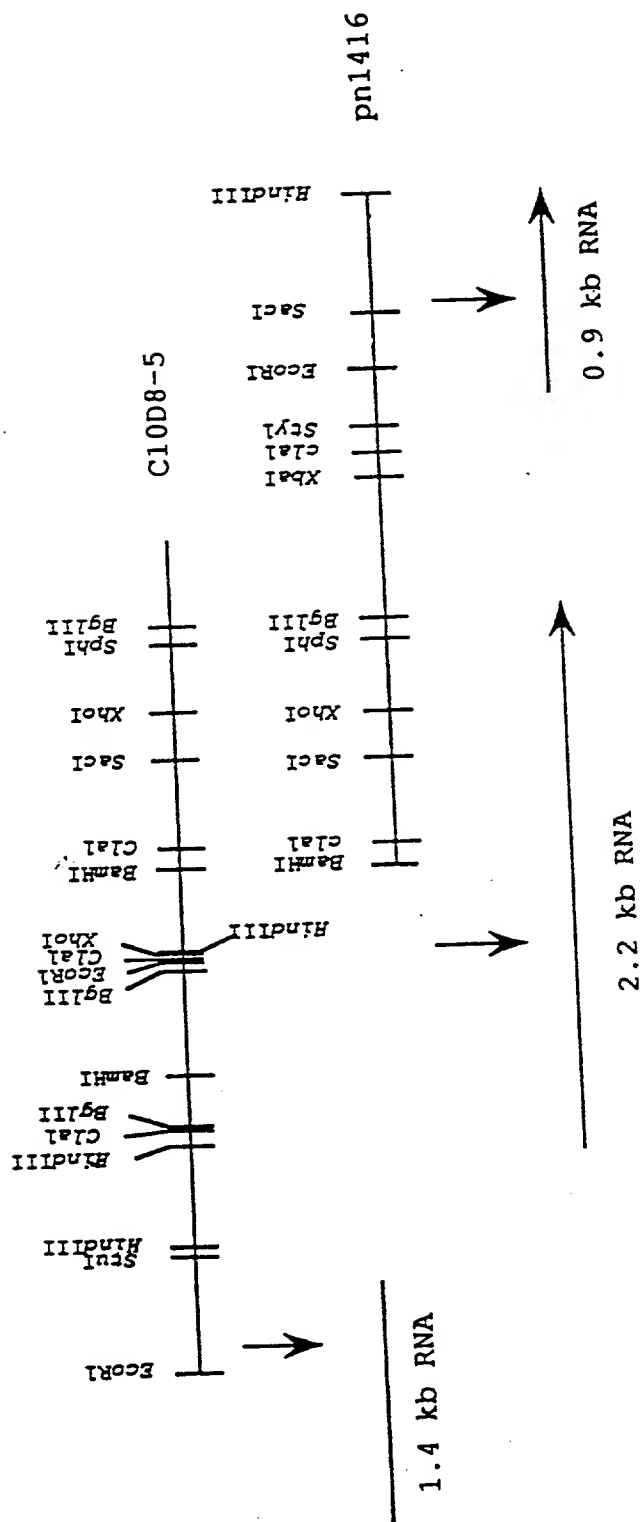


FIGURE 8

IV

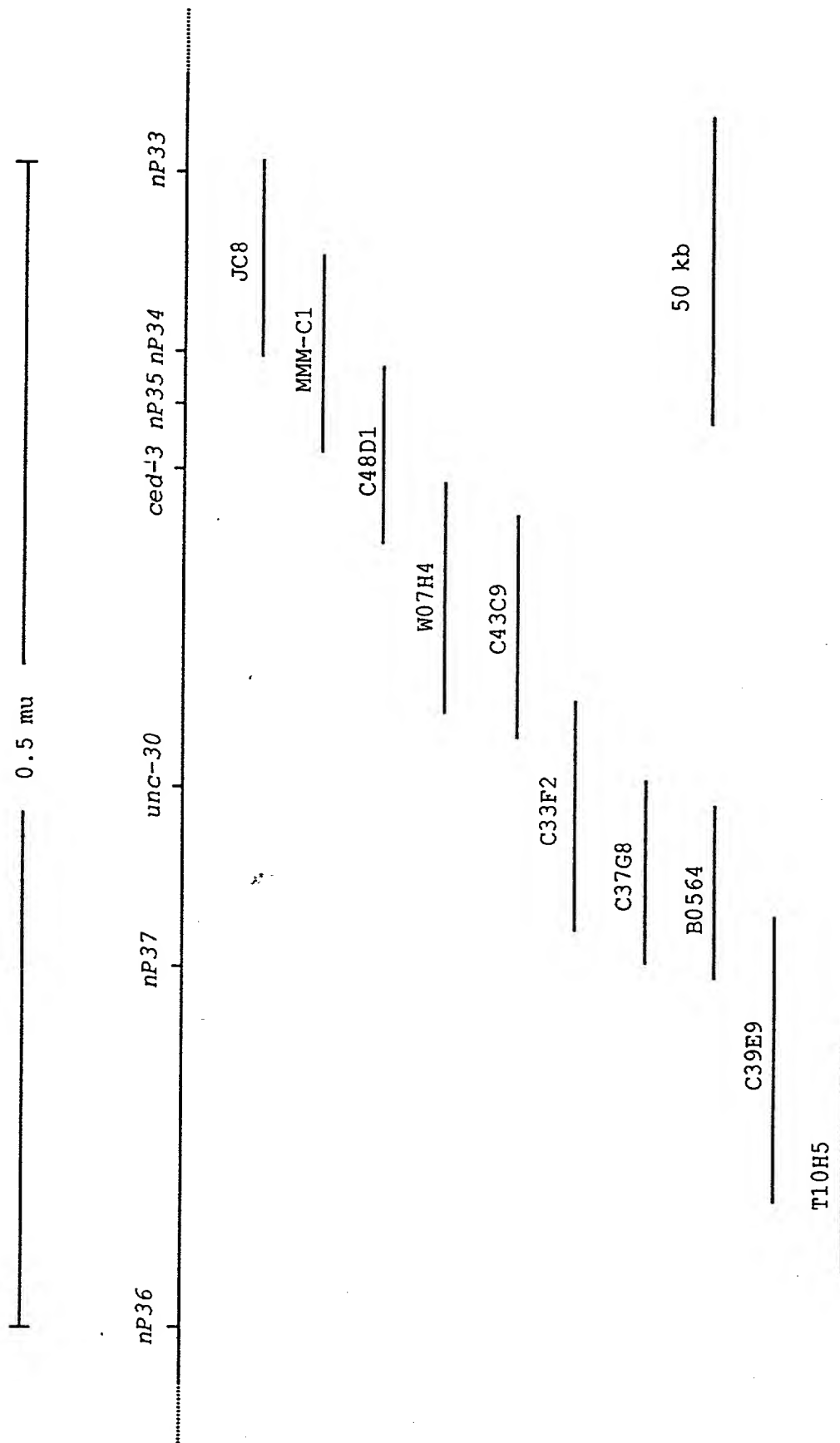


FIGURE 9

Figure 10 Summary of the experiments to localize *ced-3* gene within C48D1.

